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(51) International Patent Classification ⁶ : C12N 15/54, C12N 9/12, 5/10, C12Q 1/48, C07K 16/40, C12N 15/00, A01K 67/027		A1	(11) International Publication Number: WO 95/30326
			(43) International Publication Date: 9 November 1995 (09.11.95)
(21) International Application Number: PCT/CA95/00254		(74) Agent: BERESKIN & PARR; 40th floor, 40 King Street West, Toronto, Ontario M5H 3Y2 (CA).	
(22) International Filing Date: 28 April 1995 (28.04.95)		(81) Designated States: CA, JP, US, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).	
(30) Priority Data: 08/235,407 29 April 1994 (29.04.94) US		Published <i>With international search report. Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i>	
(60) Parent Application or Grant (63) Related by Continuation US 08/235,407 (CIP) Filed on 29 April 1994 (29.04.94)			
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(54) Title: NEURAL RECEPTOR TYROSINE KINASE			
(57) Abstract <p>Novel receptor tyrosine kinase protein and isoforms thereof which are expressed in migrating axons, and nucleic acid molecules encoding the novel protein isoforms and parts thereof are disclosed. The invention also relates to methods for identifying substances which are capable of binding to the receptor protein and methods for screening for agonists or antagonists of the binding of the protein and substance. Diagnostic and therapeutic methods using the protein and nucleic acid molecules are also described.</p>			

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Neural receptor tyrosine kinase

FIELD OF THE INVENTION

The invention relates to a novel receptor tyrosine kinase protein and isoforms and parts thereof, nucleic acid molecules encoding the novel protein and fragments thereof, and uses of the protein and nucleic acid molecules.

BACKGROUND OF THE INVENTION

Embryonic development of multicellular organisms is a highly ordered process that requires coordination of individual cells. Every cell must decipher the numerous signals it receives and then properly execute commands in order to achieve the correct position and differentiated state in the animal. The exquisite controls over cell growth, determination, migration and adhesion are mediated by molecules located on the plasma membrane surface.

A class of membrane associated molecules known to regulate cellular interactions are receptor tyrosine kinase proteins. The evolutionary conservation of genes encoding receptor tyrosine kinase proteins and their targets has emphasized the importance of these proteins in intracellular communication, and has also provided model systems for genetic analysis of tyrosine kinase signalling pathways. Such studies have shown that some tyrosine kinases function to specify a particular cell fate, such as the sevenless (sev) receptor in *Drosophila* R7 photoreceptor cells and the Let-23 receptor in nematode vulval cells (reviewed by Greenwald and Rubin, *Cell* 68:271-281, 1992). The binding of sev with its ligand, boss, results in cell clustering suggesting a role in cell-cell adhesion for these molecules (Kramer *et al.*, *Nature* 352:207-212, 1991). The receptor tyrosine kinase encoded by *torso* functions in pattern formation by specifying the terminal poles of *Drosophila* embryos (Sprenger *et al.*, *Nature* 338:478-483, 1989). Genetic analysis has recently provided insight into the functions of a small number of receptor tyrosine kinases in mouse development, including the α -platelet-derived growth factor receptor, the colony stimulating factor-1 receptor, and c-Kit/W (Pawson and Bernstein, *Trends in Genetics* 6:350-356, 1990).

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A growing number of closely related transmembrane receptor tyrosine kinase proteins containing cell adhesion-like domains on their extracellular surface have recently been identified. Collectively, this group of proteins defines the *Eph/Elk/Eck* subfamily, which is made up of at least fifteen related but unique gene sequences in higher vertebrates (Hirai *et al.*, *Science* 238:1717-1720, 1987; Letwin *et al.*, *Oncogene* 3:621-627, 1988; Lindberg *et al.*, *Mol. Cell. Biol.* 10:6316-6324, 1990; Lhotak *et al.*, *Mol. Cell. Biol.* 11:2496-2502, 1991; Chan and Watt, *Oncogene* 6:1057-1061, 1991; Lai and Lemke, *Neuron* 6:691-704, 1991; Pasquale, *Cell Regulation* 2:523-534, 1991; Sajjadi *et al.*, *New Biologist* 3:769-778, 1991; Wicks *et al.*, *PNAS* 89:1611-1615, 1992; Gilardi-Hebenstreit *et al.*, *Oncogene* 7:2499-2506, 1992; Bohme *et al.*, *Oncogene* 8:2857-2862, 1993; Sajjadi and Pasquale, *Oncogene* 8:1807-1813, 1993). *Eph* family members encode a structurally related cysteine rich extracellular domain containing a single immunoglobulin (Ig)-like loop near the N-terminus and two fibronectin III (FN III) repeats adjacent to the plasma membrane. Examples of *Eph* family members include *Cek5* (Pasquale, *Cell Regulation* 2:523-534, 1991) and *Erk*; (Chan and Watt, *Oncogene* 6:1057-1061 1991). Another *Eph* family member, *Sek*, has been shown to be segmentally expressed in specific rhombomeres of the mouse hindbrain (Nieto *et al.*, *Development* 116:1137-1150, 1992). The presence of cell adhesion-like domains in this family of tyrosine kinases suggests that these proteins function in cell-cell interactions.

The other major families of proteins implicated in cell adhesion include the cadherins, selectins, integrins, and those of the immunoglobulin superfamily (reviewed by Hynes, R.O. and Landers, A.D., *Cell* 68, 303-322, 1992). The extracellular regions of cell adhesion molecules frequently contain peptide repeats, such as FN III motifs, epidermal growth factor (EGF) repeats, or Ig loops that may direct protein-protein interactions at the cell surface. A number of cell adhesion molecules in both vertebrates (Dodd, J. and Jessell, T.M., *Science*, 242, 692-699, 1988; Jessell, T.M., *Neuron*, 1, 3-13, 1988; Furley *et al.*, *Cell* 61, 157-170, 1990; Burns *et al.*, *Neuron*, 7, 209-220, 1991) and invertebrates (Bastiani *et al.*, *Cell* 48:745-755, 1987; Elkins *et al.*, *Cell* 60:565-575,

1990; Grenningloh *et al.*, *Cold Spring Harb. Symp. Quant. Biol.* 55, 327-340, 1991; Nose *et al.*, *Cell* 70:553-567, 1992) have been implicated in axonal growth cone guidance and pathway/target recognition. Other aspects of neuronal morphogenesis involving cell-cell interactions may also require the activities of cell adhesion molecules (Edelman and Thiery, In *The Cell in Contact: Adhesions and Junctions as Morphogenetic Determinants*, Wiley, New York, 1985; Hatta *et al.*, *Dev. Biol.* 120:215-227, 1987; Takeichi, *Development* 102:639-655, 1988; Takeichi, *Annu. Rev. Biochem.* 59:237-252 1990; Takeichi, *Science* 251:1451-1455, 1991; Edelman, *Biochemistry* 27:3533-3543, 1988; Grumet, *Curr. Opin. Neurobiol.* 1:370-376, 1991; Hynes and Lander, *Cell* 68:303-322, 1992). For example, ectopic N-cadherin expression during gastrulation stage *Xenopus* embryos has been shown to interfere with segregation of the neural tube from the ectoderm (Detrick *et al.*, *Neuron* 4:493-506, 1990; Fujimori *et al.*, *Development* 110:97-104, 1990). Although many different types of cell adhesion molecules have been identified, little is known about how these adhesive interactions are regulated and how they function in cell signalling pathways during normal development.

A critical stage in the development of the nervous system is the projection of axons to their targets. Navigational decisions are made at the growth cones of the migrating axons. As axons grow their growth cones extend and retract filopodia and lamellipodia processes which are implicated in the navigational decisions and pathfinding abilities of migrating axons. Like peripheral nervous system axons, the growth cones of neurons associated with the central nervous system follow stereotyped pathways and apparently can selectively chose from a number of possible routes (reviewed by Goodman and Shatz, *Cell* 72:77-98, 1993). Early pathways in the vertebrate embryonic brain are thought to be arranged as a set of longitudinal tracts connected by commissures. However, the molecular mechanisms that underly growth cone navigation and axon pathfinding in development are poorly understood (Hynes, R.O. and Lander, A.D., 1992, *Cell* 68:303).

Evidence indicates that the development of the endolymphatic duct is under the control of neuronal induction (Van De Water and Represa, Van

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De Water, T.R. and Represa, J. (1991). *Ann. NY Acad.* 630:116-128, 1991). The endolymphatic duct pinches off from the otic vesicle and elongates to form a tube that apparently functions in regulating the endolymph fluid pressure in the membranous labyrinth of the internal ear (Guild, *Amer. J. Anat.* 39:57-81, 1927; Rugh, *The Mouse: Its reproduction and Development*. Minneapolis: Burgess, 1968; Sher, 1971; Hendriks and Toerien, 1973; Theiler, 1989; Kaufman, In *Postimplantation Mammalian Embryos: a Practical Approach* (ed. A.J. Copp and D.L. Cockroft) pp. 81-91. New York: Oxford University Press, 1990).

The developmental function of tyrosine kinases during axonogenesis has been studied in *Drosophila*. A function in axonal pathfinding is evident for the *Drosophila* *abl* tyrosine kinase when *abl* mutations are combined with mutations in other genes including the neural cell adhesion molecule, *fasciclin I* (*fas I*, Elkins *et al.*, *Cell* 60:565-575, 1990) or *disabled* (*dab*, Gertler *et al.*, *Cell* 58:103-113, 1989). These studies have shown that the *abl* tyrosine kinase is specifically localized to the axonal compartment of the embryonic Central Nervous System (CNS) (Gertler *et al.*, *Cell* 58:103-113, 1989). Moreover, genetic analysis has indicated that subcellular localization to axons is essential for *abl* function during development (Henkemeyer *et al.*, *Cell* 63:949-960, 1990) and that mutations in second-site modifier genes including *fas I* and *dab* can reveal a role for *abl* in axonogenesis (Elkins *et al.*, *Cell* 60:565-575, 1990; Gertler *et al.*, *Cell* 58:103-113 1989). The requirement for tyrosine phosphorylation in axonal outgrowth and adhesion in *Drosophila* is strengthened by the identification in CNS axons of three transmembrane tyrosine phosphatases containing FN III motifs (Tian *et al.*, *Cell* 67:675-685, 1991; Yang *et al.*, *Cell* 67:661-673, 1991).

SUMMARY OF THE INVENTION

The present inventors have identified and characterized a receptor tyrosine kinase protein that plays an important role in cell-cell interactions and axonogenesis in the development of the nervous system. In particular, the present inventors have cloned a novel murine gene, designated neural kinase (*Nuk*). The gene encodes a new member of the Eph subfamily of receptor tyrosine kinases, designated Nuk protein. The murine *Nuk* locus was

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mapped to the distal end of mouse chromosome 4 near the *ahd-1* mutation.

The biological function of Nuk protein was investigated using antibodies having anti-Nuk protein specificity. A detailed immunohistochemical analysis of its subcellular localization in whole-mount mouse embryos indicated that during early embryogenesis Nuk protein is confined to the developing nervous system, where it marks segments along the axis of the neural tube in the hindbrain (rhombomeres r2, r3, and r5) and specific morphological bulges of the midbrain and forebrain.

Nuk protein was also found to be concentrated at sites of cell-cell contact, often involving migrating neuronal cells or their extensions. Most notably, high levels of Nuk protein were found within initial axon outgrowths and associated nerve fibers, including most if not all peripheral nervous system (PNS) axons. The axonal localization of Nuk protein was also found to be transient and was not detected after migrations have ceased.

The subcellular localization of Nuk protein, as well as the presence of fibronectin type III and immunoglobulin-like adhesive domains on the extracellular region, indicates that this receptor tyrosine kinase functions to regulate specific cell-cell interactions during early development of the nervous system and that it has a role during the early pathfinding and/or fasciculation stages of axonogenesis in animals.

The present invention therefore provides a purified and isolated nucleic acid molecule containing a sequence encoding a receptor tyrosine kinase protein which is expressed in migrating axons, or an oligonucleotide fragment of the sequence which is unique to the receptor tyrosine kinase protein. In a preferred embodiment of the invention, the purified and isolated nucleic acid molecule comprises (a) a nucleic acid sequence encoding a protein having the amino acid sequence as shown in SEQ ID NO:2 and Figure 2; (b) nucleic acid sequences complementary to (a); (c) nucleic acid sequences which are at least 97% identical to (a); or, (d) a fragment of (a) or (b) that is at least 15 bases and which will hybridize to (a) or (b) under stringent hybridization conditions.

Most preferably, the purified and isolated nucleic acid molecule

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comprises (a) a nucleic acid sequence as shown in SEQ ID NO:1 and Figure 1, wherein T can also be U; (b) nucleic acid sequences complementary to (a); (c) nucleic acid sequences which are at least 85% identical to (a); or, (d) a fragment of (a) or (b) that is at least 15 bases and which will hybridize to (a) or (b) under
5 stringent hybridization conditions.

It is contemplated that a nucleic acid molecule of the invention may be prepared having deletion and insertion mutations. For example, the extracellular domain or parts thereof, such as the FN III and Ig domains; the transmembrane region or parts thereof; the tyrosine kinase domain or parts
10 thereof, such as the ATP binding site and; the carboxy terminal tail may be deleted. In a preferred embodiment, the deletions are in a portion of the nucleic acid molecule of the invention encoding the extracellular domain of Nuk protein, most preferably the portion comprising codons 29 to 50 in SEQ ID NO:1. In another preferred embodiment, the deletions are in a portion of
15 the nucleic acid sequence of the invention encoding the kinase domain of Nuk protein, most preferably the portion comprising the ATP-binding site amino acid number 623-707.

The invention further contemplates a purified and isolated double stranded nucleic acid molecule containing a nucleic acid molecule of the invention or a fragment thereof, hydrogen bonded to a complementary
20 nucleic acid base sequence.

The nucleic acid molecules of the invention, or fragments thereof may be inserted into an appropriate expression vector, i.e. a vector which contains the necessary elements for the transcription and translation of the
25 inserted protein-coding sequence. Accordingly, recombinant DNA molecules adapted for transformation of a host cell may be constructed which comprise a nucleic acid molecule of the invention and one or more transcription and translation elements operatively linked to the nucleic acid molecule.

In an embodiment of the invention, a recombinant molecule is
30 provided which contains a nucleic acid molecule of the invention having a deletion or insertion mutation. Such a recombinant molecule may further comprise a reporter gene.

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The recombinant molecule can be used to prepare transformed host cells expressing the protein or part thereof encoded by a nucleic acid molecule of the invention. Therefore, the invention further provides host cells containing a recombinant molecule of the invention. The invention also
5 contemplates transgenic non-human mammals whose germ cells and somatic cells contain a recombinant molecule of the invention.

The invention further provides a method for preparing a novel receptor tyrosine kinase protein or isoforms or parts thereof utilizing the purified and isolated nucleic acid molecules of the invention.

10 The invention further broadly contemplates a purified and isolated receptor tyrosine kinase protein which is expressed in migrating axons, or an isoform or a part of the protein. In a preferred embodiment, a purified receptor tyrosine kinase protein is provided which has the amino acid sequence as shown in SEQ ID NO:2 or Figure 2, or a sequence having between 97 and 100
15 percent identity thereto. The receptor tyrosine kinase protein of the invention may also be phosphorylated.

Conjugates of Nuk protein of the invention, or parts thereof, with other molecules, such as proteins or polypeptides, may be prepared. This may be accomplished, for example, by the synthesis of N-terminal or C-terminal
20 fusion proteins. In a preferred embodiment a fusion protein is provided comprising a part of the protein of the invention, preferably the extracellular domain, most preferably having the amino acid sequence as shown in SEQ ID NO: 2 from amino acid number 26 to 548 or amino acids 600 to 618; or the carboxy terminal, most preferably having the amino acid sequence as shown
25 in SEQ ID NO:2 from amino acid number 601 to 994; or sequences having at least 97% identity thereto.

The invention also permits the construction of nucleotide probes which are unique to the nucleic acid molecules of the invention and accordingly to the novel receptor tyrosine kinase protein of the invention or a
30 part of the protein. Thus, the invention also relates to a probe comprising a nucleotide sequence coding for a protein which displays the properties of the novel receptor tyrosine kinase of the invention or a part which is unique to

the protein. The probe may be labelled, for example, with a detectable substance and it may be used to select from a mixture of nucleotide sequences a nucleotide sequence coding for a protein which displays the properties of the novel receptor tyrosine kinase protein of the invention, or a part thereof.

5 The invention still further provides a method for identifying a substance which is capable of binding to the novel receptor tyrosine kinase protein of the invention, or an isoform or part of the protein, comprising reacting the novel receptor tyrosine kinase protein of the invention, or part of the protein, with at least one substance which potentially can bind with the
10 receptor tyrosine kinase protein, isoform or part of the protein, under conditions which permit the formation of substance-receptor kinase protein complexes, and assaying for substance-receptor kinase protein complexes, for free substance, for non-complexed receptor kinase proteins, or for activation of the receptor tyrosine kinase proteins.

15 In an embodiment of the method, ligands are identified which are capable of binding to and activating the novel receptor tyrosine kinase protein of the invention. The extracellular ligands which bind to and activate the novel receptor tyrosine kinase protein of the invention may be identified by assaying for protein tyrosine kinase activity.

20 Still further, the invention provides a method for assaying a medium for the presence of an agonist or antagonist of the interaction of a receptor tyrosine kinase protein of the invention and a substance which binds to the receptor tyrosine kinase protein, preferably a ligand. In an embodiment, the method comprises providing a known concentration of a receptor tyrosine
25 kinase protein of the invention, isoforms thereof, or part of the protein, preferably the extracellular domain of the protein, incubating the receptor tyrosine kinase protein with a substance which is capable of binding to the receptor tyrosine kinase protein, isoforms thereof, or part of the protein, and a suspected agonist or antagonist substance under conditions which permit the
30 formation of substance-receptor protein complexes, and assaying for substance-receptor protein complexes, for free substance, for non-complexed proteins, or for activation of the receptor tyrosine kinase protein. The methods

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of the invention permit the identification of potential stimulators or inhibitors of axonal migration and nerve cell interactions in development and regeneration, which will be useful in the treatment of nerve disorders and nerve damage.

5 The invention also contemplates a method for identifying a substance which is capable of binding to an activated receptor tyrosine kinase protein of the invention, or an isoform or part of the activated protein, comprising reacting the activated receptor tyrosine kinase protein of the invention, or part of the protein, with at least one substance which potentially
10 can bind with the receptor tyrosine kinase protein, isoform or part of the protein, under conditions which permit the formation of substance-receptor kinase protein complexes, and assaying for substance-receptor kinase protein complexes, for free substance, for non-complexed receptor kinase proteins, or for phosphorylation of the substance. In an embodiment of the method,
15 intracellular ligands such as Src homology region 2 (SH2)-containing proteins which are capable of binding to a phosphorylated receptor tyrosine kinase protein of the invention, or intracellular ligands which may be phosphorylated by the novel receptor tyrosine kinase of the invention may be identified.

20 The invention further contemplates antibodies having specificity against an epitope of the receptor tyrosine kinase protein of the invention or part of the protein which is unique to the receptor tyrosine kinase protein. Antibodies may be labelled with a detectable substance and they may be used to detect the novel receptor tyrosine kinase of the invention in tissues
25 and cells. The antibodies may accordingly be used to monitor axonal migration and nerve cell interactions.

 Substances which affect axonal migration may be identified using the methods of the invention by comparing the pattern and level of expression of the novel receptor tyrosine kinase protein of the invention in tissues and cells
30 in the presence and in the absence of the substance. Thus the invention provides a method for screening for substances having pharmaceutical utility in the treatment and diagnosis of nerve disorders and nerve damage.

Other objects, features and advantages of the present invention will become apparent from the following detailed description. It should be understood, however, that the detailed description and the specific examples while indicating preferred embodiments of the invention are given by way of illustration only, since various changes and modifications within the spirit and scope of the invention will become apparent to those skilled in the art from this detailed description.

DESCRIPTION OF THE DRAWINGS

The invention will be better understood with reference to the drawings in which:

Figure 1 shows the nucleotide sequence encoding the novel receptor tyrosine kinase protein of the invention as shown in SEQ ID NO: 1;

Figure 2 shows the amino acid sequence of the novel receptor tyrosine kinase protein of the invention as shown in SEQ ID NO:2 and a schematic diagram of the regions of the novel receptor tyrosine kinase protein of the invention;

Figure 3A shows immunoprecipitation of the novel receptor tyrosine kinase protein of the invention;

Figure 3B shows Western Blot analysis of immunoprecipitates of the novel receptor tyrosine kinase protein of the invention;

Figure 4A shows localization of mRNA of the novel receptor tyrosine kinase protein of the invention in whole-mount sections of 7.5 day old mouse embryo;

Figure 4B shows localization of the novel receptor tyrosine kinase protein of the invention in whole-mount sections of 8 day old mouse embryo;

Figure 4C shows localization of the novel receptor tyrosine kinase protein of the invention in whole-mount sections of 8.75 day old mouse embryo;

Figure 4D shows localization of the novel receptor tyrosine kinase protein of the invention in whole-mount sections of 9.5 day old mouse embryo;

Figure 4E shows localization of the novel receptor tyrosine kinase

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protein of the invention in whole-mount sections of 9.5 day old mouse embryo in greater detail than in Figure 4D;

Figure 4F shows localization of the novel receptor tyrosine kinase protein of the invention in paraffin serial transverse sections of 9.5 day old mouse embryo;

Figure 4G shows localization of the novel receptor tyrosine kinase protein of the invention in paraffin serial transverse sections of 9.5 day old mouse embryo;

Figure 4H shows localization of the novel receptor tyrosine kinase protein of the invention in paraffin sagittal section along the midline of 10.5 day old mouse embryo;

Figure 4I shows localization of the novel receptor tyrosine kinase protein of the invention in paraffin adjacent transverse sections of 10.5 day old mouse embryo immunoreacted with anti-Nuk protein antibodies;

Figure 4J shows localization of the novel receptor tyrosine kinase protein of the invention in paraffin adjacent transverse sections of 10.5 day old mouse embryo immunoreacted with a trpE-Nuk peptide;

Figure 5A shows an adjacent transverse section of an 11.5 day embryo at the level of the caudal/posterior spinal cord immunoreacted with anti-Nuk antibodies;

Figure 5B shows an adjacent transverse section of an 11 day embryo at the level of the caudal/posterior spinal cord immunoreacted with anti-Nuk antibodies preincubated with a trpE-Nuk peptide;

Figure 5C shows transmission electron microscopy immunolocalization of Nuk protein in ventral midbrain cells of a 9.5 day embryo;

Figure 5D shows transmission electron microscopy (EM) immunolocalization of Nuk protein in ventral midbrain cells of a 9.5 day embryo;

Figure 6A is a photomicrograph of the head of an 11.5 day anti-Nuk whole-mount immunostained embryo with one of the pair of oculomotor nerve fibers in focus showing strong labelling for Nuk protein (filled arrow);

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Figure 6B shows a clearer view of Nuk protein staining in the oculomotor axons fibers obtained when the whole-mount staged embryo was filleted down the midline to minimize tissue thickness;

Figure 6C shows a paraffin section of a 10.5 to 11 day mouse embryo
5 immunostained with anti-Nuk;

Figure 6D shows a paraffin section of a 10.5 to 11 day mouse embryo immunostained with anti-Neurofilament antibodies.

Figure 6E is a frontal section immunostained with anti-Nuk antibodies which label the ventral midbrain and oculomotor axon fibers as
10 they exit the neural tube (filled arrow) and extend (open arrow) towards their target tissue, the pre-optic muscle mass;

Figure 6F shows a sagittal section immunostained with anti-Nuk antibodies which label the oculomotor axon fascicle as it enters the pre-optic muscle mass (open arrow);

Figure 7A is a 10.5 day whole-mount embryo immunostained with
15 anti-Nuk antibodies which label the trigeminal nerve V and facial nerve VII;

Figures 7B shows transverse sections of a 10.5 day embryo showing Nuk-positive trigeminal nerve V axon fascicles labelled with the anti-Nuk antibodies;

Figures 7C shows transverse sections of a 10.5 day embryo showing
20 Nuk-positive trigeminal nerve V axon fascicles labelled with the anti-Nuk antibodies;

Figures 7D is an 11.5 day anti-Nuk whole-mount showing localization of Nuk protein in the vagus nerve X associated fibers as they
25 pathfind to their target visceral organs (curved open arrows);

Figures 7E is an 11.5 day anti-Nuk whole-mount showing localization of Nuk protein in the vagus nerve X associated fibers as they pathfind to their target visceral organs (curved open arrows);

Figure 7F is a whole-mount 10 day embryo showing Nuk protein
30 concentrated within the earliest spinal nerve fibers exiting the neural tube (arrows);

Figure 7G shows a slightly later stage embryo from that shown in

Figure 7F;

Figures 7H shows a transverse section bisecting the rostral spinal cord of an 11 day embryo demonstrating that the darkly stained Nuk-positive fibers shown in Figures 7F and 7G are ventral motor axons (open arrows).

5 Figures 7I shows a transverse section bisecting the rostral spinal cord of an 11 day embryo demonstrating that the darkly stained Nuk-positive fibers shown in Figures 7F and 7G are ventral motor axons (open arrows).

10 Figures 7J shows both sides of an 11 day whole-mount embryo demonstrating that Nuk protein is localized within the spinal motor nerves as they elongate to the plexus regions (open arrows);

Figures 7K shows both sides of an 11 day whole-mount embryo demonstrating that Nuk protein is localized within the spinal motor nerves as they elongate to the plexus regions;

15 Figure 7L shows a close-up of a 12 day whole-mount embryo immunostained with anti-Nuk antibodies which label the nerve fibers of the parasympathetic chain ganglion;

Figure 8A shows a whole-mount 10.5 day embryo showing Nuk protein localization (arrow) at the dorsal region of the otic vesicle (ov) surrounding the budding endolymphatic duct;

20 Figure 8B shows a whole-mount 11.5 day embryo showing elongation of the endolymphatic duct;

25 Figure 8C shows a whole-mount 11.5 day embryo at a slightly different focal plane from Figure 8B showing that the vestibulocochlear sensory fibers connecting to the developing ear stain positive for Nuk protein (arrow);

Figure 8D shows a transverse section of an 11.5 day embryo showing high levels of Nuk protein localized to the basement membrane of the endolymphatic duct cells where they contact the surrounding mesenchymal cells (open arrow);

30 Figure 9 shows a recombinant DNA molecule of the invention having a null mutation obtained by deletion of exon 2, corresponding to codons 29 to 50 as shown in SEQ ID NO: 1;

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Figure 10 shows a recombinant DNA molecule of the invention encoding the *Nuk¹* mutation in the ATP binding region of the kinase domain of Nuk protein, and a lac Z reporter gene;

5 Figure 11A shows expression of the *Nuk²* mutation in a mouse embryo at the six somite stage (8.25 days development) in the brain and developing heart;

Figure 11B shows expression of the *Nuk²* mutation in a mouse embryo at the 14 somite stage (8.75 days development) in the hindbrain rhombomeres, the midbrain, diencephalon and in the heart (B)

10 Figure 12A shows *Nuk²* expression in a 10.5 day old mouse embryo in the ventral midbrain, diencephalon and retinal cells;

Figure 12B shows *Nuk²* expression in a 10.5 day old mouse embryo in the brain and spinal cord;

15 Figure 13 shows an immunoblot illustrating that Nuk protein autophosphorylation is induced by Elk-ligand stimulation;

Figure 14 is an immunoblot showing expression of Nuk protein in cell lines;

20 Figure 15 is an immunoblot showing binding of phosphorylated/non-phosphorylated Nuk protein to SH2-containing GST-fusion proteins;

Figure 16 is an immunoblot showing binding of phosphorylated/non-phosphorylated Nuk protein to SH2-containing GST-fusion proteins;

25 Figure 17 is an immunoblot showing binding of Nuk Protein to SH2-containing GST-fusion proteins in the presence of a competing phosphorylated peptide;

Figure 18 shows immunoblots illustrating the phosphorylation of proteins after ELK-ligand stimulation of COS cells.

DETAILED DESCRIPTION OF THE INVENTION

30 I. Characterization of Nucleic Acid Molecules and Proteins of the Invention

As hereinbefore mentioned, the present inventors have identified and sequenced a nucleic acid molecule encoding a novel receptor tyrosine

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kinase protein with a unique expression pattern as described herein. The receptor tyrosine kinase protein of the invention is also referred to herein as Neural Kinase (Nuk) protein.

5 The *Nuk* coding region was cloned using a λ gt10 expression library constructed from mouse embryo mRNA. The library was probed with a partial n Q1 *Nuk* cDNA insert. Additional 5'*Nuk* coding sequences were obtained by rapid amplification of cDNA ends (RACE). Translation of combined RACE and cDNA clones revealed a single open reading frame of 994 codons.

10 The *Nuk* locus mapped to the distal end of mouse chromosome 4 near the *ahd*-1 mutation. The Nuk protein belongs to the *Eph/Elk/Eck* family, of which many members are expressed in the developing nervous system. The protein encoded by the deduced amino acid sequence of Nuk has all the hallmarks of an Eph family member, including a number of conserved residues of the Eph family, for example the 20 cysteine residues whose
15 position is conserved in the extracellular domain of Eph family members (bold type, Figure 2), an immunoglobulin-like domain near the amino terminus (Ig-like), and two fibronectin type III repeats (FN III); between Nuk residues 330-420 and 444-534. The Ig-like domain of Nuk protein contains specific residues (Cys⁷⁰, Trp⁸⁰, Cys¹¹⁵) known to be conserved in the Ig superfamily
20 (Williams and Barclay, *Ann. Rev. Immunol.* 6:381-405, 1988). When compared to other known members of the Eph family, Nuk protein was found to be most highly related to the full length amino acid sequence of chicken Cek5 (96% identity).

25 In accordance with an embodiment of the invention a purified and isolated nucleic acid molecule is provided containing a sequence encoding a protein having the amino acid sequence as shown in SEQ ID NO:2 and Figure 2. Most preferably, the purified and isolated nucleic acid molecule of the invention contains a nucleic acid sequence as shown in SEQ ID NO:1 and Figure 1.

30 Fragments of the nucleic acid molecules are contemplated by the present invention. In a preferred embodiment, the fragments include fragments of the nucleotide sequence as shown in SEQ. ID. NO. 1 and in

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Figure 1 that have at least 15 bases to 18 bases, preferably at least 15 bases, and which are capable of hybridizing to the nucleotide sequence as shown in SEQ ID NO. 1 and Figure 1 under stringent hybridization conditions as described herein. These fragments may encode, for example, the extracellular domain
5 (amino acids 26 to 548, SEQ ID NO:2) or the carboxy tail (amino acids 601 to 994, SEQ ID NO:2).

It will also be appreciated that a double stranded nucleotide sequence comprising a nucleic acid molecule of the invention or a fragment thereof, hydrogen bonded to a complementary nucleotide base sequence, and an RNA
10 made by transcription of this double stranded nucleotide sequence are contemplated by the present invention.

Further, it will be appreciated that the invention includes nucleic acid or amino acid sequences which have substantial sequence identity with the nucleic acid and amino acid sequences shown in SEQ ID NOS:1 and 2 and
15 in Figures 1 and 2, and fragments thereof. The term "sequences having substantial sequence identity" means those nucleic acid and amino acid sequences which have slight or inconsequential sequence variations from the sequences disclosed in Figures 1 and 2 and SEQ ID NOS: 1 and 2, i.e. the homologous sequences function in substantially the same manner to produce
20 substantially the same polypeptides as the actual sequences. The variations may be attributable to local mutations or structural modifications.

Nucleic acid sequences having substantial identity include nucleic acid sequences which encode proteins having at least 97% sequence identity with the amino acid sequences as shown in SEQ. ID. NO:2 and in Figure 2;
25 nucleic acid sequences having at least 85%, preferably at least 90%, most preferably at least 95% identity with the nucleic acid sequence as shown in SEQ. ID. NO.:1 and in Figure 1; and fragments thereof having at least 15 to 18, preferably at least 15 bases which will hybridize to these sequences under stringent hybridization conditions. Stringent hybridization conditions are
30 those which are stringent enough to provide specificity, reduce the number of mismatches and yet are sufficiently flexible to allow formation of stable hybrids at an acceptable rate. Such conditions are known to those skilled in the

art and are described, for example, in Sambrook, et al, (1989, Molecular Cloning, A Laboratory Manual, Cold Spring Harbor). By way of example only, stringent hybridization with short nucleotides may be carried out at 5-10° below the T_m using high concentrations of probe such as 0.01-1.0pmole/ml.

5 The invention further provides amino acid sequences which have substantial identity with the amino acid sequence shown in SEQ ID NO:2 and in Figure 2. Substantially identical sequences include sequences having at least 97% sequence identity. The invention still further provides peptides which are unique to the receptor tyrosine kinase protein of the invention. Preferably, the
10 peptides have at least 10 to 20 amino acids.

 The sequence of the nucleic acid molecule of the invention or a fragment thereof, may be inverted relative to its normal presentation for transcription to produce antisense nucleic acid molecules. The antisense nucleic acid molecules may be constructed using chemical synthesis and
15 enzymatic ligation reactions using procedures known in the art. The antisense nucleic acid molecules may be used in gene therapy to treat inherited disorders of the nervous system.

 A number of unique restriction sequences for restriction enzymes are incorporated in the nucleic acid sequence identified in SEQ ID NO: 1 and in
20 Figure 1 and these provide access to nucleic acid sequences which code for polypeptides unique to the receptor tyrosine kinase protein of the invention. Nucleic acid sequences unique to the receptor tyrosine kinase protein of the invention or isoforms or parts thereof, can also be constructed by chemical synthesis and enzymatic ligation reactions carried out by procedures known in
25 the art.

 The invention contemplates isoforms of the receptor tyrosine kinase protein of the invention. An isoform contains the same number and kinds of amino acids as the protein of the invention, but the isoform has a different molecular structure. The isoforms contemplated by the present invention are
30 those having the same functional properties as the novel receptor tyrosine kinase protein of the invention as described herein.

 The present invention also includes conjugates of the receptor

tyrosine kinase protein of the invention, or parts thereof. For example, the receptor tyrosine kinase protein or portions thereof may be conjugated with a selected protein or marker protein to produce fusion proteins.

5 The present invention also includes a receptor tyrosine kinase protein of the invention or part thereof, preferably the catalytic domain, which is enzymatically active. The catalytically active form of the protein or part thereof is also referred to herein as an "activated receptor tyrosine kinase protein or part thereof".

10 II. Expression Pattern of the Receptor Tyrosine Kinase Protein of the Invention

The expression of Nuk protein has been localized during early embryogenesis. The restricted expression of Nuk imposes constraints on the cellular range of activity of the putative Nuk ligand, and indicates that the *Nuk* locus plays unique and important roles in the determination, migration
15 and pathfinding of axons, in axogenesis and fasciculation, in neural tube formation, and in the regulation of specific cell-cell interactions during early development of the nervous system. As many features of regeneration in the peripheral nervous system recapitulate development, for example, following injury and wallerian degeneration, axons regrow and migrate to reinnervate
20 their targets, the *Nuk* locus also plays an important role in axonal migration during regeneration following injury to the peripheral nervous system.

The present inventors have shown that, in the early stages of embryonic development, Nuk protein is confined to the developing nervous system, where it marks segments along the axis of the neural tube in the
25 hindbrain and specific morphological bulges of the midbrain and forebrain. Nuk is expressed in a rhombomere-specific pattern early during hindbrain segmentation. The restriction of Nuk protein to specific anterior-posterior and dorsal-ventral compartments during early development of the rostral neural tube indicates this receptor tyrosine kinase protein functions in the patterning
30 of specific brain structures.

Nuk protein was found to be expressed in the developing nervous system and, in particular, is highly expressed very early in the retinal ganglion

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cells and in the group of cells that form the optic chiasm just prior to axonogenesis of the retinal cells. These observations indicate that Nuk protein participates in early development of the visual system components and in pathfinding of retinal axons.

5 The present inventors have also detected Nuk in cells of the ventral midbrain and in the endolymphatic duct of the developing ear.

 The present inventors have also localized Nuk protein to specific locations within the cells of the developing nervous system and have shown that Nuk protein is associated with the plasma membrane of migrating neural
10 cells. In particular, Nuk protein is concentrated at sites of cell-cell contact, of migrating neuronal cells or their extensions and high levels of Nuk protein are found within initial axon outgrowths and associated nerve fibers. The axonal localization of Nuk is transient and is not detected after the growth cones have reached their targets and migrations have ceased, indicating a role
15 for this receptor tyrosine kinase protein during the early migration, pathfinding and fasciculation stages of axonogenesis. Nuk protein may function to transmit signals from the plasma membrane and may cooperate with other neuronal tyrosine kinases, such as Sek.

 The present inventors found that Nuk protein is localized to at least
20 one CNS axon pathway. The subcellular localization of Nuk protein is similar to that observed for vimentin and the extracellular matrix molecule laminin (Liesi, *EMBO* 4:1163-1170, 1985) and it coincides with pathways of neuronal cell migration along the radial glial fibers (Hatten, *Trends Neurosci.* 13:179-184, 1990).

25 The specific subcellular localization of Nuk protein to the cell-cell contacts between the basement membrane of the endolymphatic duct cells and the surrounding mesenchyme/neural crest cells indicates Nuk protein functions to modulate this interaction.

 The concentration of Nuk protein at sites of cell-cell contact indicates
30 that its ligand is a membrane-associated molecule. In addition, Nuk protein immunoreactivity was frequently observed on the membranes of both cells at the site of contact and was generally observed to localize to specific regions of

the membrane (see Figures 5 and 8). Therefore, homophilic/heterophilic interactions between Eph receptors may play a role in their biological functions.

III. Preparation of Nucleic Acid Molecules and Proteins of the Invention

5 The nucleic acid molecules of the invention encoding the novel receptor tyrosine kinase protein, or fragments thereof, may be isolated and sequenced, for example, by synthesizing cDNAs from mouse embryo RNA and using rapid amplification of cDNA ends (RACE, Frohman, et al., Proc. Na+, Acad. Sci. USA, 85, 8998-9002, 1988) using oligonucleotides specific for the
10 novel receptor tyrosine kinase protein, and analysing the sequences of the clones obtained following amplification. Oligonucleotides specific for the novel receptor tyrosine kinase protein may be identified by comparing the nucleic acid sequence of the nucleic acid molecules of the invention to known sequences, for example, sequences of the other members of the *Eph* subfamily.
15 Nucleic acid molecules of the present invention encoding the novel receptor tyrosine kinase protein and oligonucleotide fragments thereof, may also be constructed by chemical synthesis and enzymatic ligation reactions using procedures known in the art.

 The novel tyrosine kinase receptor protein of the invention may be
20 prepared using recombinant DNA methods. Accordingly, the nucleic acid molecules of the present invention having a sequence which codes for the receptor tyrosine kinase protein of the invention, or a fragment thereof may be incorporated in a known manner into an appropriate expression vector which ensures good expression of the protein or part thereof. Possible
25 expression vectors include but are not limited to cosmids, plasmids, or modified viruses, so long as the vector is compatible with the host cell used.

 The invention therefore contemplates a recombinant molecule of the invention containing a nucleic acid molecule of the invention, or a fragment thereof, and the necessary elements for the transcription and
30 translation of the inserted protein-sequence. Suitable transcription and translation elements may be derived from a variety of sources, including bacterial, fungal, viral, mammalian, or insect genes. Selection of appropriate

transcription and translation elements is dependent on the host cell chosen as discussed below, and may be readily accomplished by one of ordinary skill in the art. Examples of such elements include: a transcriptional promoter and enhancer or RNA polymerase binding sequence, a ribosomal binding
5 sequence, including a translation initiation signal. Additionally, depending on the host cell chosen and the vector employed, other genetic elements, such as an origin of replication, additional DNA restriction sites, enhancers, and sequences conferring inducibility of transcription may be incorporated into the expression vector. It will also be appreciated that the necessary transcriptional
10 and translation elements may be supplied by the native receptor tyrosine kinase protein and/or its flanking regions.

The recombinant molecules of the invention may also contain a reporter gene which facilitates the selection of host cells transformed or transfected with a recombinant molecule of the invention. Examples of
15 reporter genes are genes encoding a protein such as β -galactosidase, chloramphenicol acetyltransferase, firefly luciferase, or an immunoglobulin or portion thereof such as the Fc portion of an immunoglobulin preferably IgG. In a preferred embodiment, the reporter gene is *lac Z*. Transcription of the reporter gene is monitored by changes in the concentration of the reporter
20 protein such as β -galactosidase, chloramphenicol acetyltransferase, or firefly luciferase. This makes it possible to visualize and assay for expression of recombinant molecules of the invention and in particular to determine the effect of a mutation on expression and phenotype.

Recombinant molecules can be introduced into host cells via
25 transformation, transfection, infection, electroporation etc. Methods for transforming transfecting, etc. host cells to express foreign DNA are well known in the art (see, e.g., Itakura et al., U.S. Patent No. 4,704,362; Hinnen et al., PNAS USA 75:1929-1933, 1978; Murray et al., U.S. Patent No. 4,801,542; Upshall et al., U.S. Patent No. 4,935,349; Hagen et al., U.S. Patent No. 4,784,950;
30 Axel et al., U.S. Patent No. 4,399,216; Goeddel et al., U.S. Patent No. 4,766,075; and Sambrook et al. Molecular Cloning A Laboratory Manual, 2nd edition,

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Cold Spring Harbor Laboratory Press, 1989, all of which are incorporated herein by reference and see the detailed discussion below).

Suitable host cells include a wide variety of prokaryotic and eukaryotic host cells, including bacterial, mammalian, yeast or other fungi, viral, plant, or insect cells.

More particularly, bacterial host cells suitable for carrying out the present invention include *E. coli*, *B. subtilis*, *Salmonella typhimurium*, and various species within the genus' *Pseudomonas*, *Streptomyces*, and *Staphylococcus*, as well as many other bacterial species well known to one of ordinary skill in the art. Representative examples of bacterial host cells include DH5n (Stratagene, LaJolla, California), JM109 ATCC No. 53323, HB101 ATCC No. 33694, and MN294. Suitable bacterial expression vectors preferably comprise a promoter which functions in the host cell, one or more selectable phenotypic markers, and a bacterial origin of replication. Representative promoters include the β -lactamase (penicillinase) and lactose promoter system (see Chang et al., Nature 275:615, 1978), the trp promoter (Nichols and Yanofsky, Meth in Enzymology 101:155, 1983) and the tac promoter (Russell et al., Gene 20: 231, 1982). Representative selectable markers include various antibiotic resistance markers such as the kanamycin or ampicillin resistance genes. Suitable expression vectors include but are not limited to bacteriophages such as lambda derivatives or plasmids such as pBR322 (see Bolivar et al., Gene 2:95, 1977), the pUC plasmids pUC18, pUC19, pUC118, pUC119 (see Messing, Meth in Enzymology 101:20-77, 1983 and Vieira and Messing, Gene 19:259-268, 1982), and pNH8A, pNH16a, pNH18a, and Bluescript M13 (Stratagene, La Jolla, Calif.).

Yeast and fungi host cells suitable for carrying out the present invention include, among others *Saccharomyces cerevisiae*, the genera *Pichia* or *Kluyveromyces* and various species of the genus *Aspergillus*. Suitable expression vectors for yeast and fungi include, among others, YC_p50 (ATCC No. 37419) for yeast, and the amdS cloning vector pV3 (Turnbull, Bio/Technology 7:169, 1989). Protocols for the transformation of yeast are also well known to those of ordinary skill in the art. For example, transformation

may be readily accomplished either by preparation of spheroplasts of yeast with DNA (see Hinnen et al., PNAS USA 75:1929, 1978) or by treatment with alkaline salts such as LiCl (see Itoh et al., J. Bacteriology 153:163, 1983). Transformation of fungi may also be carried out using polyethylene glycol as described by Cullen et al. (Bio/Technology 5:369, 1987).

Mammalian cells suitable for carrying out the present invention include, among others: COS (e.g., ATCC No. CRL 1650 or 1651), BHK (e.g., ATCC No. CRL 6281), CHO (ATCC No. CCL 61), HeLa (e.g., ATCC No. CCL 2), 293 (ATCC No. 1573) and NS-1 cells. Suitable expression vectors for directing expression in mammalian cells generally include a promoter, as well as other transcriptional and translational control sequences. Common promoters include SV40, MMTV, metallothionein-1, adenovirus Ela, CMV, immediate early, immunoglobulin heavy chain promoter and enhancer, and RSV-LTR. Protocols for the transfection of mammalian cells are well known to those of ordinary skill in the art. Representative methods include calcium phosphate mediated electroporation, retroviral, and protoplast fusion-mediated transfection (see Sambrook et al., supra).

Given the teachings provided herein, promoters, terminators, and methods for introducing expression vectors of an appropriate type into plant, avian, and insect cells may also be readily accomplished. For example, within one embodiment, *Nuk* or derivatives thereof may be expressed from plant cells (see Sinkar et al., J. Biosci (Bangalore) 11:47-58, 1987, which reviews the use of Agrobacterium rhizogenes vectors; see also Zambryski et al., Genetic Engineering, Principles and Methods, Hollaender and Setlow (eds.), Vol. VI, pp. 253-278, Plenum Press, New York, 1984, which describes the use of expression vectors for plant cells, including, among others, pAS2022, pAS2023, and pAS2034).

Insect cells suitable for carrying out the present invention include cells and cell lines from *Bombyx* or *Spodoptera* species. Suitable expression vectors for directing expression in insect cells include Baculoviruses such as the *Autographa californica* nuclear polyhedrosis virus (Miller et al. 1987, in *Genetic Engineering*, Vol. 8 ed. Setler, J.K. et al., Plenum Press, New York) and

the *Bombyx mori* nuclear polyhedrosis virus (Maeda et al., 1985, Nature 315:592).

Alternatively, *Nuk* may be expressed in non-human transgenic animals such as, mice, rats, rabbits, sheep and pigs (see Hammer et al. (Nature 5 315:680-683, 1985), Palmiter et al. (Science 222:809-814, 1983), Brinster et al. (Proc Natl. Acad. Sci USA 82:44384442, 1985), Palmiter and Brinster (Cell. 41:343-345, 1985) and U.S. Patent No. 4,736,866).

The *Nuk* protein or parts thereof may also be prepared by chemical synthesis using techniques well known in the chemistry of proteins such as 10 solid phase synthesis (Merrifield, 1964, J. Am. Chem. Assoc. 85:2149-2154) or synthesis in homogenous solution (Houbenweyl, 1987, Methods of Organic Chemistry, ed. E. Wansch, Vol. 15 I and II, Thieme, Stuttgart).

Conjugates of the *Nuk* protein of the invention, or parts thereof, with other molecules, such as proteins or polypeptides, may be prepared. This 15 may be accomplished, for example, by the synthesis of N-terminal or C-terminal fusion proteins. Thus, fusion proteins may be prepared by fusing, through recombinant techniques, the N-terminal or C-terminal of *Nuk* protein or parts thereof, and the sequence of a selected protein or marker protein with a desired biological function. The resultant fusion proteins 20 contain *Nuk* protein or a portion thereof fused to the selected protein or marker protein as described herein. Examples of proteins which may be used to prepare fusion proteins include immunoglobulins and parts thereof such as the constant region of immunoglobulin γ 1, and lymphokines such as gamma interferon, tumor necrosis factor, IL-1, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, 25 IL-10, IL-11, GM-CSF, CSF-1 and G-CSF.

Sequences which encode the above-described proteins may generally be obtained from a variety of sources, including for example, depositories which contain plasmids encoding sequences including the American Type Culture Collection (ATCC, Rockville Maryland), and the British 30 Biotechnology Limited (Cowley, Oxford England). Examples of such plasmids include BBG 12 (containing the GM-CSF gene coding for the mature protein of 127 amino acids), BBG 6 (which contains sequences encoding gamma

interferon), ATCC No. 39656 (which contains sequences encoding TNF), ATCC No. 20663 (which contains sequences encoding alpha interferon,) ATCC Nos. 31902 and 39517 (which contains sequences encoding beta interferon), ATCC No. 67024 (which contains a sequence which encodes Interleukin-1 β), ATCC
5 Nos. 39405, 39452, 39516, 39626 and 39673 (which contains sequences encoding Interleukin-2), ATCC Nos. 59399, 59398, and 67326 (which contain sequences encoding Interleukin-3), ATCC Nos. 57592 (which contains sequences encoding Interleukin-4). ATCC Nos. 59394 and 59395 (which contain sequences encoding Interleukin-5), and ATCC No. 67153 (which contains sequences
10 encoding Interleukin-6).

Within a particularly preferred embodiment of the invention, *Nuk* is cloned into an expression vector as a fusion gene with the constant region of human immunoglobulin γ 1. Briefly, the expression vectors pNUTAGH and pVL1393 are prepared for cloning by digestion with *Sma*I followed by
15 dephosphorylation by calf intestinal alkaline phosphatase. The linear product is isolated after agarose gel electrophoresis. The *Nuk* genes are then generated by polymerase chain reaction using the cloned *Nuk* cDNA as a template. In particular, the *Nuk* fusion protein is synthesized from the extracellular domain of *Nuk* protein, preferably amino acids 26 to 548, SEQ ID NO: 2 and
20 Figure 2. In another embodiment, the *Nuk* fusion protein is synthesized from the carboxy terminal tail of *Nuk* protein, preferably amino acids 601 to 994, SEQ ID NO:2 and Figure 2.

The constant region of an immunoglobulin, such as human γ 1 gene may be prepared, for example, from pUCB7Ig monomer. Briefly, the C_H gene is
25 isolated by digestion with *Xba*I which cuts at the 3' end of the gene followed by treatment with *E. coli* DNA polymerase I in the presence of all four dNTPs in order to create a blunt end. The plasmid is then digested with *Bcl*I which cuts at the 5' end of the gene. The fragment containing the heavy chain gene is isolated after electrophoresis in an agarose gel.

30 The fusion *Nuk* amplified fragment is inserted into each prepared vector along with the heavy chain fragment. Orientation of the resulting

plasmids is determined by PCR with one priming oligo which anneals to the vector sequence and the other priming oligo which anneals to the insert sequence. Alternatively, appropriate restriction digests can be performed to verify the orientation. The sequence of the fusion *Nuk*/immunoglobulin constant region gene can be verified by DNA sequencing.

Phosphorylated receptor tyrosine kinase proteins of the invention, or parts thereof, may be prepared using the method described in Reedijk et al. The EMBO Journal 11(4):1365, 1992. For example, tyrosine phosphorylation may be induced by infecting bacteria harbouring a plasmid containing a nucleotide sequence of the invention or fragment thereof, with a λ gt11 bacteriophage encoding the cytoplasmic domain of the Elk tyrosine kinase. Bacteria containing the plasmid and bacteriophage as a lysogen are isolated. Following induction of the lysogen, the expressed receptor protein becomes phosphorylated.

IV. Utility of the Nucleic Acid Molecules and Proteins of the Invention

The nucleic acid molecules of the invention or fragments thereof, allow those skilled in the art to construct nucleotide probes for use in the detection of nucleotide sequences in biological materials. A nucleotide probe may be labelled with a detectable substance such as a radioactive label which provides for an adequate signal and has sufficient half-life such as ^{32}P , ^3H , ^{14}C or the like. Other detectable substances which may be used include antigens that are recognized by a specific labelled antibody, fluorescent compounds, enzymes, antibodies specific for a labelled antigen, and luminescent compounds. An appropriate label may be selected having regard to the rate of hybridization and binding of the probe to the nucleotide to be detected and the amount of nucleotide available for hybridization. Labelled probes may be hybridized to nucleic acids on solid supports such as nitrocellulose filters or nylon membranes as generally described in Sambrook et al, 1989, Molecular Cloning, A Laboratory Manual (2nd ed.). The nucleotide probes may be used to detect genes, preferably in human cells, that encode proteins related to or analogous to the receptor tyrosine kinase protein of the invention. The nucleotide probes may therefore be useful in the diagnosis of disorders of the

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nervous system arising from mutations or alterations to the *Nuk* gene or a homologue thereof.

5 The receptor tyrosine kinase protein of the invention and portions thereof, for example amino acids of the carboxy terminal tail, preferably amino acids 601 to 994; or amino acids of the extracellular domain, preferably amino acids 26 to 548 (SEQ ID NO: 2 and Figure 2), may be used to prepare antibodies. Antibodies having specificity for Nuk protein may also be raised from fusion proteins created by expressing trpE-Nuk fusion proteins in bacteria as described above.

10 Within the context of the present invention, antibodies are understood to include monoclonal antibodies, polyclonal antibodies, antibody fragments (e.g., Fab, and F(ab')₂ and recombinantly produced binding partners. Antibodies are understood to be reactive against Nuk protein if they bind with a K_a of greater than or equal to 10⁻⁷ M. As will be appreciated by one of
15 ordinary skill in the art, antibodies may be developed which not only bind to Nuk protein, but which bind to a ligand of Nuk protein, and which also block the biological activity of Nuk protein. Such antibodies will be useful in the diagnosis and treatment of disorders of the nervous system and nerve damage.

20 Polyclonal antibodies may be readily generated by one of ordinary skill in the art from a variety of warm-blooded animals such as horses, cows, various fowl, rabbits, mice, or rats. Briefly, Nuk protein is utilized to immunize the animal through intraperitoneal, intramuscular, intraocular, or subcutaneous injections, in conjunction with an adjuvant such as Freund's
25 complete or incomplete adjuvant. Following several booster immunizations, samples of serum are collected and tested for reactivity to Nuk protein. Particularly preferred polyclonal antisera will give a signal on one of these assays that is at least three times greater than background. Once the titer of the animal has reached a plateau in terms of its reactivity to Nuk protein, larger
30 quantities of antisera may be readily obtained either by weekly bleedings, or by exsanguinating the animal.

Monoclonal antibodies may also be readily generated using

conventional techniques (see U.S. Patent Nos. RE 32,011, 4,902,614, 4,543,439, and 4,411,993 which are incorporated herein by reference; see also Monoclonal Antibodies, Hybridomas: A New Dimension in Biological Analyses, Plenum Press, Kennett, McKearn, and Bechtol (eds.), 1980, and Antibodies: A Laboratory Manual, Harlow and Lane (eds.), Cold Spring Harbor Laboratory Press, 1988, which are also incorporated herein by reference).

Briefly, within one embodiment a subject animal such as a rat or mouse is injected with Nuk protein. The Nuk protein may be admixed with an adjuvant such as Freund's complete or incomplete adjuvant in order to increase the resultant immune response. Between one and three weeks after the initial immunization the animal may be reimmunized with another booster immunization, and tested for reactivity to Nuk protein using assays described above. Once the animal has plateaued in its reactivity to Nuk protein, it is sacrificed, and organs which contain large numbers of B cells such as the spleen and lymph nodes are harvested.

Cells which are obtained from the immunized animal may be immortalized by transfection with a virus such as the Epstein bar virus (EBV) (see Glasky and Reading, Hybridoma 8(4):377-389, 1989). Alternatively, within a preferred embodiment, the harvested spleen and/or lymph node cell suspensions are fused with a suitable myeloma cell in order to create a "hybridoma" which secretes monoclonal antibody. Suitable myeloma lines include, for example, NS-1 (ATCC No. TIB 18), and P3X63 - Ag 8.653 (ATCC No. CRL 1580).

Following the fusion, the cells may be placed into culture plates containing a suitable medium, such as RPMI 1640, or DMEM (Dulbecco's Modified Eagles Medium) (JRHI Biosciences, Lenexa, Kansas), as well as additional ingredients, such as Fetal Bovine Serum (FBS, ie., from Hyclone, Logan, Utah, or JRHI Biosciences). Additionally, the medium should contain a reagent which selectively allows for the growth of fused spleen and myeloma cells such as HAT (hypoxanthine, aminopterin, and thymidine) (Sigma Chemical Co., St. Louis, Missouri). After about seven days, the resulting fused cells or hybridomas may be screened in order to determine the presence of

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antibodies which are reactive against Nuk protein. A wide variety of assays may be utilized to determine the presence of antibodies which are reactive against Nuk protein, including for example Countercurrent Immuno-Electrophoresis, Radioimmunoassays, Radioimmunoprecipitations, 5 Enzyme-Linked Immuno-Sorbent Assays (ELISA), Dot Blot assays, Inhibition or Competition Assays, and sandwich assays (see U.S. Patent Nos. 4,376,110 and 4,186,530; see also Antibodies: A Laboratory Manual, Harlow and Lane (eds.), Cold Spring Harbor Laboratory Press, 1988). Following several clonal dilutions and reassays, a hybridoma producing antibodies reactive against Nuk 10 protein may be isolated.

Other techniques may also be utilized to construct monoclonal antibodies (see William D. Huse et al., "Generation of a Large Combinational Library of the Immunoglobulin Repertoire in Phage Lambda," Science 246:1275-1281, December 1989; see also L. Sastry et al., "Cloning of the 15 Immunological Repertoire in Escherichia coli for Generation of Monoclonal Catalytic Antibodies: Construction of a Heavy Chain Variable Region-Specific cDNA Library," Proc Natl. Acad. Sci USA 86:5728-5732, August 1989; see also Michelle Alting-Mees et al., "Monoclonal Antibody Expression Libraries: A Rapid Alternative to Hybridomas," Strategies in Molecular Biology 3:1-9, 20 January 1990; these references describe a commercial system available from Stratacyte, La Jolla, California, which enables the production of antibodies through recombinant techniques). Briefly, mRNA is isolated from a B cell population, and utilized to create heavy and light chain immunoglobulin cDNA expression libraries in the λ ImmunoZap(H) and λ ImmunoZap(L) 25 vectors. These vectors may be screened individually or co-expressed to form Fab fragments or antibodies (see Huse et al. supra; see also Sastry et al., supra). Positive plaques may subsequently be converted to a non-lytic plasmid which allows high level expression of monoclonal antibody fragments from *E. coli*.

Similarly, binding partners may also be constructed utilizing 30 recombinant DNA techniques to incorporate the variable regions of a gene which encodes a specifically binding antibody. Within one embodiment, the genes which encode the variable region from a hybridoma producing a

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monoclonal antibody of interest are amplified using nucleotide primers for the variable region. These primers may be synthesized by one of ordinary skill in the art, or may be purchased from commercially available sources. Primers for mouse and human variable regions including, among others, primers for
5 V_{Ha}, V_{Hb}, V_{Hc}, V_{Hd}, C_{H1}, V_L and C_L regions are available from Stratacyte (La Jolla, Calif). These primers may be utilized to amplify heavy or light chain variable regions, which may then be inserted into vectors such as ImmunoZAP™H or ImmunoZAP™L (Stratacyte), respectively. These vectors may then be introduced into *E. coli* for expression. Utilizing these techniques,
10 large amounts of a single-chain protein containing a fusion of the VH and VL domains may be produced (See Bird et al., Science 242:423-426, 1988). In addition, such techniques may be utilized to change a "murine" antibody to a "human" antibody, without altering the binding specificity of the antibody.

Once suitable antibodies or binding partners have been obtained,
15 they may be isolated or purified by many techniques well known to those of ordinary skill in the art (see Antibodies: A Laboratory Manual, Harlow and Lane (eds.), Cold Spring Harbor Laboratory Press, 1988). Suitable techniques include peptide or protein affinity columns, HPLC or RP-HPLC, purification on protein A or protein G columns, or any combination of these techniques.

20 The polyclonal or monoclonal antibodies may be used to detect the receptor tyrosine kinase protein of the invention in various biological materials, for example they may be used in an ELISA, radioimmunoassay or histochemical tests. Thus, the antibodies may be used to quantify the amount of a receptor tyrosine kinase protein of the invention in a sample in order to
25 determine its role in particular cellular events or pathological states and to diagnose and treat such pathological states.

In particular, the polyclonal and monoclonal antibodies of the invention may be used in immuno-histochemical analyses, for example, at the cellular and sub-subcellular level, to detect the novel receptor tyrosine
30 kinase protein of the invention, to localise it to particular cells and tissues and to specific subcellular locations, and to quantitate the level of expression.

Cytochemical techniques known in the art for localizing antigens

using light and electron microscopy may be used to detect the novel tyrosine kinase of the invention. Generally, an antibody of the invention may be labelled with a detectable substance and the novel receptor tyrosine kinase of the invention may be localised in tissue based upon the presence of the detectable substance. Examples of detectable substances include various enzymes, fluorescent materials, luminescent materials and radioactive materials. Examples of suitable enzymes include horseradish peroxidase, biotin, alkaline phosphatase, β -galactosidase, or acetylcholinesterase; examples of suitable fluorescent materials include umbelliferone, fluorescein, fluorescein isothiocyanate, rhodamine, dichlorotriazinylamine fluorescein, dansyl chloride or phycoerythrin; an example of a luminescent material includes luminol; and examples of suitable radioactive material include radioactive iodine I¹²⁵, I¹³¹ or tritium. Antibodies may also be coupled to electron dense substances, such as ferritin or colloidal gold, which are readily visualised by electron microscopy.

Radioactive labelled materials may be prepared by radiolabeling with ¹²⁵I by the chloramine-T method (Greenwood et al, Biochem. J. 89:114, 1963), the lactoperoxidase method (Marchalonis et al, Biochem. J. 124:921, 1971), the Bolton-Hunter method (Bolton and Hunter, Biochem. J. 133:529, 1973 and Bolton Review 18, Amersham International Limited, Buckinghamshire, England, 1977), the iodogen method (Fraker and Speck, Biochem. Biophys. Res. Commun. 80:849, 1978), the Iodo-beads method (Markwell Anal. Biochem. 125:427, 1982) or with tritium by reductive methylation (Tack et al., J. Biol. Chem. 255:8842, 1980).

Known coupling methods (for example Wilson and Nakane, in "Immunofluorescence and Related Staining Techniques", W. Knapp et al, eds, p. 215, Elsevier/North-Holland, Amsterdam & New York, 1978; P. Tijssen and E. Kurstak, Anal. Biochem. 136:451, 1984) may be used to prepare enzyme labelled materials. Fluorescent labelled materials may be prepared by reacting the material with umbelliferone, fluorescein, fluorescein isothiocyanate, dichlorotriazinylamine fluorescein, dansyl chloride, derivatives of rhodamine such as tetramethyl rhodamine isothiocyanate, or phycoerythrin.

Indirect methods may also be employed in which the primary antigen-antibody reaction is amplified by the introduction of a second antibody, having specificity for the antibody reactive against the novel tyrosine kinase of the invention. By way of example, if the antibody having specificity
5 against the novel tyrosine kinase protein of the invention is a rabbit IgG antibody, the second antibody may be goat anti-rabbit gamma-globulin labelled with a detectable substance as described herein.

Where a radioactive label is used as a detectable substance, the novel tyrosine kinase of the invention may be localized by radioautography. The
10 results of radioautography may be quantitated by determining the density of particles in the radioautographs by various optical methods, or by counting the grains.

As discussed above, the expression patterns found for the novel tyrosine kinase of the invention indicate that it plays unique and important
15 roles in the determination, migration and pathfinding of axons, in axogenesis and fasciculation, development and regeneration of the neural tube, and in the regulation of specific cell-cell interactions during early development of the nervous system. Therefore, the above described methods for detecting nucleic acid molecules and fragments thereof and Nuk protein and parts thereof, can
20 be used to monitor vertebrate axonal migration, fasciculation and regeneration by detecting and localizing the novel tyrosine kinase protein of the invention in migrating axons and in the migrating membrane surface of cells of the developing nervous system.

It would also be apparent to one skilled in the art that the above
25 described methods may be used to study the developmental expression of *Nuk* and, accordingly, will provide further insight into the role of Nuk protein in neuronal cell:cell interactions in embryonic development and in axonogenesis and nerve regeneration.

The finding of a novel receptor tyrosine kinase protein which is
30 expressed in migrating axons and the developing neural tube permits the identification of substances which may affect axonogenesis, neural embryonic development and neuron regeneration. A substance which affects expression

of Nuk protein may be assayed using the above described methods for detecting nucleic acid molecules and fragments thereof and Nuk protein and parts thereof, by comparing the pattern and level of expression of the Nuk protein or parts thereof, in the presence and absence of the substance.

5 The invention also provides methods for identifying substances which are capable of binding to the Nuk protein, or isoforms and parts thereof. In particular, the methods may be used to identify ligands and natural and synthetic derivatives of such ligands, which are capable of binding to and in some cases activating the receptor tyrosine kinase protein of the invention,
10 isoforms thereof, or part of the protein.

 Substances which can bind with the receptor tyrosine kinase protein of the invention may be identified by reacting the novel receptor tyrosine kinase protein which is expressed in migrating vertebrate axons isoforms thereof, or part of the protein, with a substance which potentially binds to the
15 novel receptor tyrosine kinase protein, isoforms thereof, or part of the protein such as the extracellular domain, and assaying for substance-receptor complexes, for free substance or for non-complexed receptor tyrosine kinase protein isoforms thereof or part of the protein, or for activation of the receptor tyrosine kinase protein.

20 Conditions which permit the formation of substance-receptor protein complexes may be selected having regard to factors such as the nature and amounts of the substance and the receptor protein.

 The substance-receptor complex, free substance or non-complexed proteins may be isolated by conventional isolation techniques, for example,
25 salting out, chromatography, electrophoresis, gel filtration, fractionation, absorption, polyacrylamide gel electrophoresis, agglutination, or combinations thereof. To facilitate the assay of the components, antibody against the receptor protein or the substance, or a labelled receptor protein, or a labelled substance may be utilized. Antibodies, receptor protein or substance may be
30 labelled with a detectable substance as described above.

 The receptor tyrosine kinase protein, isoforms or parts thereof, or substance used in the method of the invention may be insolubilized. For

example, the receptor protein or substance may be bound to a suitable carrier. Examples of suitable carriers are agarose, cellulose, dextran, Sephadex, Sepharose, carboxymethyl cellulose polystyrene, filter paper, ion-exchange resin, plastic film, plastic tube, glass beads, polyamine-methyl vinyl-ether-maleic acid copolymer, amino acid copolymer, ethylene-maleic acid copolymer, nylon, silk, etc. The carrier may be in the shape of, for example, a tube, test plate, beads, disc, sphere etc.

The insolubilized receptor tyrosine kinase protein or substance may be prepared by reacting the material with a suitable insoluble carrier using known chemical or physical methods, for example, cyanogen bromide coupling.

The receptor tyrosine kinase protein, parts thereof, or substance may also be expressed on the surface of a cell using the methods described herein.

The above mentioned methods of the invention may be used to identify ligands which bind with and activate the novel receptor tyrosine kinase protein of the invention thereby affecting signalling pathways, particularly those involved in neuronal development and axonal migration and regeneration. Identification and isolation of such a Nuk protein ligand will permit studies of the role of the ligand in the developmental regulation of axonogenesis and neural regeneration, and permit the development of substances which affect these roles, such as functional or non-functional analogues of the ligand. It will be appreciated that such substances will be useful as pharmaceuticals to modulate axonogenesis, nerve cell interactions and regeneration to treat conditions such as neurodegenerative diseases and cases of nerve injury.

Ligands which bind to and activate the novel receptor tyrosine kinase protein of the invention may be identified by assaying for protein tyrosine kinase activity i.e. by assaying for phosphorylation of the tyrosine residues of the novel receptor tyrosine kinase protein.

Receptor tyrosine kinase protein activity may be assayed using known techniques such as those using anti-phosphotyrosine antibodies and labelled phosphorous. For example, immunoblots of the complexes may be

analyzed by autoradiography (^{32}P -labelled samples) or may be blocked and probed with antiphosphotyrosine antibodies as described in Koch, C.A. et al (1989) Mol. Cell. Biol. 9, 4131-4140.

5 The ligands for many receptor tyrosine kinase proteins are cell-bound, either as they are associated with the cell surface via heparin and hepatocyte growth factor or because they are transmembrane proteins (Lyman et al. 1993, supra). Ligands for receptor tyrosine kinases of the *Eph/Elk/Eck* subfamily of receptor tyrosine kinases may require cell-to-cell contact to activate the receptor. Membrane attachment of the ligand could facilitate
10 ligand dimerization or clustering, or both, which may promote receptor multimerization and activation.

Accordingly, a ligand for Nuk protein may have a cell-bound form. A cell-bound ligand may be identified by reacting the receptor tyrosine kinase protein of the invention, an isoform or a part thereof with a cell suspected of
15 expressing the ligand on the surface of the cell following the procedures generally described in Lyman et al., 1993, (Cell 75:1157-1167). Thus, the invention provides a method for identifying cells expressing a surface bound ligand of Nuk protein and for specifically selecting for such cells.

By way of example, a cDNA encoding a ligand for Nuk protein may
20 be cloned by first constructing a fusion protein. The fusion protein may consist of the extracellular domain of Nuk protein (amino acids 26 to 548, SEQ ID NO: 2 and Figure 2). The fusion protein may be expressed and used as a probe to examine cells or cell lines (e.g. neuroblastoma or neuroepithelioma cell lines) for their capacity to bind the extracellular domain of Nuk protein (determined
25 by flow cytometry). The identification of cells and cell lines that bind the extracellular domain may be facilitated by incorporating in the fusion protein a sequence encoding a marker protein for example, the Fc portion of human IgG which may be detected with labelled anti-human IgG antibodies. Cells or cell lines which bind the extracellular domain are presumed to express a
30 cell-bound form of the ligand.

Following identification of a source of the Nuk ligand, a cDNA expression library is constructed, following known techniques, using mRNA

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from the cells/cell lines which have been identified as binding the fusion protein containing the extracellular domain of Nuk protein. cDNAs are then transfected into host cells (e.g. COS cells and see discussion herein re host cells) which are then screened for their capacity to bind the extracellular domain of Nuk protein. Individual clones which are capable of binding the extracellular domain of Nuk protein are identified and the cDNAs are sequenced. The cDNAs may be used as hybridization probes to isolate genomic DNA encoding the ligand.

The invention also contemplates a method for assaying for an agonist or antagonist of the binding of the novel receptor tyrosine kinase of the invention with a substance which is capable of binding with the novel tyrosine kinase protein, preferably a ligand. The agonist or antagonist may be an endogenous physiological substance or it may be a natural or synthetic drug. Substances which are capable of binding with the Nuk protein and preferably ligands, including cell-bound ligands may be identified using the methods set forth herein. Substances which bind to other receptor tyrosine kinases of the *Eph/Elk/Eck* subfamily may also be used in this assay. For example, the substance may be LERK-2, a binding protein for the receptor-tyrosine kinase ELK (Fletcher, F.A. et al., *Oncogene* (1994), 9, 3241-3247), or the cell-bound ligands B61 (also known as EFL-1), EHK1-L (also known as EFL-2) and ELK-L (also known as EFL-3) (Davis, S. et al., *Science* Vol. 266, p.816, Nov. 4, 1994).

In accordance with a preferred embodiment, a method is provided which comprises providing a known concentration of the novel receptor tyrosine kinase protein of the invention, incubating the protein with a ligand which can bind to and activate the protein, and a suspected agonist or antagonist under conditions which permit the formation of substance-receptor protein complexes, and assaying for substance-receptor protein complexes, for free substance, for non-complexed proteins, or for activation of the receptor tyrosine kinase protein. Conditions which permit the formation of substance-receptor protein complexes, and methods for assaying for substance-receptor protein complexes, for free substance, for non-complexed

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proteins, or for activation of the receptor tyrosine kinase protein are described herein.

It will be understood that the agonists and antagonists that can be assayed using the methods of the invention may act on one or more of the
5 binding sites on the receptor tyrosine kinase or the ligand, including agonist binding sites, competitive antagonist binding sites, non-competitive antagonist binding sites or allosteric sites.

The invention also makes it possible to screen for antagonists that inhibit the effects of an agonist of the interaction of Nuk protein with a Nuk
10 protein ligand. Thus, the invention may be used to assay for a substance that competes for the same ligand binding site of the novel receptor tyrosine kinase protein of the invention.

The invention further contemplates a method for identifying a substance which is capable of binding to an activated receptor tyrosine kinase
15 protein of the invention or an isoform or part of the activated protein, comprising reacting an activated receptor tyrosine kinase protein of the invention, or an isoform, or part of the protein, with at least one substance which potentially can bind with the receptor tyrosine kinase protein, isoform
20 or part of the protein, under conditions which permit the formation of substance-receptor kinase protein complexes, and assaying for substance-receptor kinase protein complexes, for free substance, for non-complexed receptor kinase proteins, or for phosphorylation of the substance.

An activated receptor tyrosine kinase protein of the invention, or
25 isoform or part thereof may be prepared by binding of a ligand to the extracellular domain of a receptor tyrosine kinase protein of the invention which results in activation of the catalytic domain. Such a ligand may be identified using the methods hereinbefore described. An activated receptor or
30 part thereof, may also be prepared using the methods described for example in Reedijk et al. The EMBO Journal, 11(4):1365, 1992 for producing a tyrosine phosphorylated receptor or part thereof.

Conditions which permit the formation of substance-receptor

protein complexes may be selected having regard to factors such as the nature and amounts of the substance and the receptor protein. The substance-receptor complex, free substance or non-complexed proteins may be isolated by conventional isolation techniques described above. Phosphorylation of the substance may be determined using for example, labelled phosphorous as described above.

In an embodiment of this method, intracellular ligands such as Src homology region 2 (SH2)-containing proteins which are capable of binding to a phosphorylated activated receptor tyrosine kinase protein of the invention may be identified. SH2-containing proteins refers to proteins containing a Src homology region 2 which is a noncatalytic domain of ~100 amino acids which was originally identified in the Vfps and Vsrc cytoplasmic tyrosine kinases by virtue of its effects on both catalytic activity and substrate phosphorylation (T. Pawson, *Oncogene* 3, 491 (1988) and I. Sadowski et al., *Mol. Cell. Biol.* 6, 4396 (1986)). (See also Koch et al., *Science* 252:668, 1991; Moran et al., *PNAS USA* 87:8622 and Anderson et al., *Science* 250:979, 1990 for discussions on SH2-containing proteins and the role of SH2 domains). SH2-containing proteins may function downstream of the Nuk signalling pathway by binding to the activated receptor protein. For example, the cytoplasmic tyrosine kinases of the Src family may bind via their SH2 domains to the activated Nuk receptor protein thereby regulating cellular processes particularly in the nervous system. Intracellular ligands which may be phosphorylated by the novel receptor tyrosine kinase of the invention may also be identified using the method of the invention.

SH2-domains of cytoplasmic signalling proteins have been found to bind to the phosphorylated receptor tyrosine kinase protein of the invention. In particular, the SH2 domains of p21^{ras} GTPase-activating protein (GAP), Src, and phosphoinositide-specific phospholipase C (PLC γ) have been found to bind Nuk protein. The SH-2 domain binding site on the Nuk protein is a conserved tyrosine containing region which is located adjacent to the membrane and it corresponds to amino acids 600 to 618 as shown in SEQ. ID. NO:2 in the Sequence Listing.

Therefore, the invention also contemplates a method for assaying for an agonist or antagonist of the binding of an activated receptor tyrosine kinase of the invention, or a portion thereof with an SH2 domain of an intracellular ligand. The agonist or antagonist may be an endogenous physiological substance or it may be a natural or synthetic drug. The activated receptor may be prepared as described herein or a portion of the ligand comprising the amino acid sequence 600 to 618 as shown in SEQ. ID. NO:2 in the Sequence Listing may be used in this method of the invention. Examples of SH2 domains of intracellular ligands include the SH2 domains of GAP, Src, and PLC γ . It will be appreciated that the entire intracellular ligand may be used in this method.

It has also been found that COS cells express large amounts of Nuk protein. Accordingly, COS cells may be used to identify *in vivo*, intracellular proteins or ligands which bind to the Nuk protein.

The invention further provides a method for assaying for a substance that affects axonal migration, neural development, nerve cell interactions and nerve regeneration comprising administering to a non-human animal or to a tissue of an animal, a substance suspected of affecting axonal migration, and detecting, and optionally quantitating, the novel receptor tyrosine kinase of the invention in the non-human animal or tissue. In an embodiment of the invention, the method may be used to assay for a substance that affects axonal migration during embryogenesis. The novel receptor tyrosine kinase of the invention may be quantitated using the methods described herein.

In another embodiment, the method may be used to assay for a substance that affects axonal migration in nerve regeneration, comprising administering a substance suspected of affecting axonal migration to a non-human animal having an injured peripheral nervous system and detecting, and optionally quantitating, the novel receptor tyrosine kinase of the invention in the non-human animal. Examples of non-human animals having an injured peripheral nervous system include animals having damaged axons, such as axotomized facial neurons (Sendtner et al. Nature,

345, 440-441, 1990), neurodegenerative conditions (for example, the MPTP model as described in Langston J.W. et al., Symposium of Current Concepts and Controversies in Parkinson's Disease, Montebello, Quebec, Canada, 1983 and Tatton W.G. et al., Can. J. Neurol. Sci. 1992, 19), and traumatic and
5 non-traumatic peripheral nerve damage (for example, animal stroke models such as the one described in MacMillan et al. Brain Research 151:353-368 (1978)).

Substances which are capable of binding to the Nuk protein of the invention or parts thereof, particularly ligands, and agonists and antagonists
10 of the binding of ligands and Nuk protein, identified by the methods of the invention, may be used for stimulating or inhibiting neuronal development, regeneration and axonal migration. The ligands, agonists and antagonists may accordingly be used to stimulate or inhibit neuronal development, regeneration and axonal migration associated with neurodegenerative
15 conditions and conditions involving trauma and injury to the nervous system, for example Alzheimer's disease, Parkinson's disease, Huntington's disease, demyelinating diseases, such as multiple sclerosis, amyotrophic lateral sclerosis, bacterial and viral infections of the nervous system, deficiency diseases, such as Wernicke's disease and nutritional polyneuropathy,
20 progressive supranuclear palsy, Shy Drager's syndrome, multistem degeneration and olivo ponto cerebellar atrophy, peripheral nerve damage, trauma and ischemia resulting from stroke.

The invention also provides methods for studying the function of the Nuk protein. Cells, tissues, and non-human animals lacking in *Nuk*
25 expression or partially lacking in *Nuk* expression may be developed using recombinant molecules of the invention having specific deletion or insertion mutations in the *Nuk* gene. For example, the extracellular domain or parts thereof, such as the FN III and Ig domains; the transmembrane region or parts thereof; the tyrosine kinase domain or parts thereof, such as the ATP binding
30 site and; the carboxy terminal tail may be deleted. A recombinant molecule may be used to inactivate or alter the endogenous gene by homologous recombination, and thereby create a *Nuk* deficient cell, tissue or animal.

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Null alleles may be generated in cells, such as embryonic stem cells by deletion mutation. A recombinant *Nuk* gene may also be engineered to contain an insertion mutation which inactivates *Nuk*. Such a construct may then be introduced into a cell, such as an embryonic stem cell, by a technique
5 such as transfection, electroporation, injection etc. Cells lacking an intact *Nuk* gene may then be identified, for example by Southern blotting, Northern Blotting or by assaying for expression of *Nuk* protein using the methods described herein. Such cells may then be fused to embryonic stem cells to generate transgenic non-human animals deficient in *Nuk*. Germline
10 transmission of the mutation may be achieved, for example, by aggregating the embryonic stem cells with early stage embryos, such as 8 cell embryos, *in vitro*; transferring the resulting blastocysts into recipient females and; generating germline transmission of the resulting aggregation chimeras. Such a mutant animal may be used to define specific nerve cell populations, developmental
15 patterns of axonogenesis, neural tube formation and nerve regeneration and *in vivo* processes, normally dependent on *Nuk* expression.

The present inventors have generated a loss of function deletion mutation in *Nuk*, designated *Nuk*¹ in mouse embryonic stem cells, and have achieved germline transmission of this null allele. In particular, the *Nuk*
20 mutation was obtained by deletion of exon 2, corresponding to codons 29 to 50, as shown in Figure 9. Adult animals homozygous for the mutation did not produce any *Nuk* protein.

A second targeted mutation, designated *Nuk*² was generated in the *Nuk* gene as shown in Figure 10 using the *pPNT-LOX-Nuk*² gene trap vector
25 to delete the GXGXXG ATP binding region of the kinase domain (amino acids 623-707, SEQ ID NO:2 and Figure 2) and to create a *Nuk-lac Z* fusion receptor. Chimeric animals expressing *Nuk*² were prepared. Animals generated with the *Nuk*² mutation provided *Nuk*² expressing cells staining for β -galactosidase activity, providing a convenient marker for *Nuk*-positive cells in both
30 heterozygous and homozygous backgrounds as detected by a blue/green colour as shown in Figures 11 and 12.

The invention also provides methods for preparing cells, tissues,

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and non-human animals lacking in *Nuk* expression or partially lacking in *Nuk* expression, and deficient in the expression of other genes. In accordance with one embodiment, an animal may be generated which is deficient in *Nuk* and another tyrosine kinase of the *Eph/Elk/Eck* subfamily. Such animals could be used to determine how the members of the *Eph/Elk/Eck* subfamily co-operate in embryonic development, particularly development of the nervous system. For example, an animal lacking or partially lacking *Nuk* expression and *Sek-4* expression (Becker N., et al., Mechanisms of Development 47 (1994) 3-17) may be generated to determine how the receptor tyrosine kinases co-operate in the segmental patterning of the hindbrain.

Multiple deficient mice can also be generated to study the interaction of *Nuk* protein and other proteins such as the *Src*-family of cytoplasmic tyrosine kinases. For example, an animal may be generated which lacks or partially lacks *Nuk* expression, and expression of one or more *Src* family tyrosine kinases including *Src*, *Fyn*, and/or *Yes*.

The following non-limiting examples are illustrative of the present invention:

EXAMPLES

The following materials and methods were utilized in the investigations outlined in the examples:

cDNA cloning, sequencing and chromosomal mapping

To clone the *Nuk* coding region, the partial λ Q1 *Nuk* cDNA insert described in Ben-David *et al.*, 1991 (EMBO 10:317) was used to probe a λ gt10 cDNA library constructed from 12.5 day mouse embryo mRNA (Logan *et al.*, Dev. Genet. 13:345-358, 1992). Sequence analysis of a number of independent clones revealed that none reached as far as the ATG translation initiation codon. To obtain additional 5' *Nuk* coding sequences, rapid amplification of cDNA ends (RACE; Frohman *et al.*, Proc. Nat. Acad. Sci. USA 85:8998-9002, 1988) was performed using *Nuk*-specific oligonucleotides. 9.5 day embryonic poly(A)⁺ RNA was used for the RACE because Northern blot analysis determined this to be a source of abundant 4.0 and 4.5 Kb *Nuk* mRNA

transcripts. A number of *Nuk* cDNA clones were generated with this approach, however, a strong stop in the majority of the RACE products just 5' of an *NheI* restriction site prevented cloning of the complete coding region. One clone out of over 50 examined (pNukRACE 2.15) extended far enough
5 such that an oligonucleotide could be used to colony screen further RACE products. Using this approach two independent clones out of over 1000 screened (pNukRACE A2 and K2) were both found to contain an additional 67 base pairs of *Nuk* coding sequences 5' of the *NheI* site. Translation of combined RACE and cDNA clones revealed a single open reading frame of 993
10 codons. Although lacking an ATG translation initiation codon, the sequence of the RACE clones encode a 26 amino acid hydrophobic signal peptide. The 5'-most sequence of both RACE clones end with the dinucleotide TG. Given that the length of the *Nuk* signal peptide is longer than most Eph family members, this TG may represent the last two nucleotides of the ATG initiator
15 codon.

The DNAs generated in this study were subcloned into either the pGEM7 (Promega) or pCRII (Invitrogen) plasmid vectors prior to double stranded sequencing using the Sequenase system (United States Biochemical Corporation). Sequencing reactions were primed using either the standard
20 forward and reverse primers or custom oligonucleotides synthesized in house on a Pharmacia Gene Assembler Plus.

Chromosome mapping was performed by probing *Pst* I restriction endonulcease digested DNAs from 28 different recombinant inbred mouse strains at high stringency with a 1.0 Kb *EcoRI* fragment of the λ Q1 *Nuk* cDNA.

25 Generation of antibodies

A *trpE*-*Nuk* fusion protein was created by subcloning a *NcoI*-*BamHI* fragment of λ Q1 containing the carboxy terminal 94 *Nuk* codons plus 170 additional nucleotides of 3' untranslated sequences into the bacterial expression vector pATH1. A GST-*Nuk* fusion protein containing *Nuk* amino
30 acids 601-994 was also expressed in bacteria. The *trpE*-*Nuk* fusion protein was purified from induced bacterial cultures by SDS-PAGE and used to immunize

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rabbits. Resulting trpE-Nuk antiserum was affinity-purified by binding to immobilized glutathione agarose purified GST-Nuk.

Immunoprecipitation, in vitro kinase, and western blotting

For biochemical studies, natural matings of CD1 mice were used to obtain embryos at 10.5, 12.5, and 14.5 days development. The embryos were collected and washed twice in phosphate buffer saline (PBS; 150 mM NaCl, 3 mM KCl, 9 mM Na₂HPO₄·2H₂O, and 2 mM KH₂PO₄) prior to homogenization in PLC-lysis buffer on ice (50 mM Hepes, pH 7.5, 150 mM NaCl, 10% glycerol, 1% Triton X-100, 1.5 mM MgCl₂, 1 mM EGTA, 10 mM NaPPi, 100 mM NaF, 10 µg/ml aprotinin and leupeptin, 1 mM Na₃VO₄, and 1 mM PMSF). The embryo lysates were clarified by centrifugation at 12,000 x rpm for 10 min at 4°C. The cleared supernatant was put on ice and an aliquot was used for protein assay (BCA protein assay, PIERCE). For immunoprecipitations, 1mg of total protein in 1 ml PLC-lysis buffer were incubated with approximately 4 mg of affinity-purified anti-Nuk antibody and 100 µl of a 10% solution of protein A-sepharose beads. Preimmune serum, as well as preincubation of the anti-Nuk antibodies with a competing trpE-Nuk peptide, were used as controls in the immunoprecipitations. After 1 hour incubation at 4°C, the immunoprecipitates were collected by centrifugation at 12,000 x rpm for 30sec and washed three times with ice cold HNTG buffer (20 mM Hepes, pH 7.5, 150 mM NaCl, 0.1% Triton X-100, 10% glycerol, and 1 mM Na₃VO₄).

For *in vitro* kinase assays, the washed immunoprecipitates were incubated at room temperature (RT) for 15 minutes in 20 µl of kinase buffer (20 mM HEPES, pH7.5, 25 mM MgCl₂, 4 mM MnCl₂, and 0.1 mM Na₃VO₄) containing 10 µCi of γ³²P-ATP (Dupont; 3000 Ci/mmol). The immune complex kinase reaction products were denatured at 100°C for 5 min in SDS sample buffer and separated by SDS-polyacrylamide gel electrophoresis. Gels were fixed in acetic acid, submerged in 1 M KOH at 55°C for 45 min to remove phosphoserine and phosphothreonine, refixed, dried, and then exposed to Kodak XAR film.

For Western blotting, the washed immunoprecipitates were separated by SDS-polyacrylamide gel electrophoresis and electro-transferred to

a nylon filter using a semi-dry protein blotting apparatus. The filter was blocked overnight at 4°C in PBS containing 5% dry milk prior to incubation in the same solution containing 1 µg/ml anti-Nuk antibodies for 1hr at room temperature (RT). The filter was washed at RT 3 X10 min with TBSN (20 mM TrisHCl, pH 7.5, 150 mM NaCl, and 0.05% NP-40). Nuk immunoreactivity was detected by using affinity-purified goat anti-rabbit horseradish peroxidase (BioRad, 1:20,000 dilution) and Enhanced Chemi-Luminescence reagents (Amersham). The filters were exposed at RT to Kodak XRP-5 film for 30 sec to 5 min.

10 Whole-Mount Immunohistochemistry

The immunohistochemical detection used in this study was based on the Vectastain ABC Elite-peroxidase system and Vectastain biotin/avidin blocking reagents (Vector Laboratories). The specificity of the anti-Nuk antibody staining was confirmed by a variety of control experiments including those in which the primary antibody was either omitted or preabsorbed with a trpE-Nuk peptide. In addition, other antibodies including a monoclonal antibody raised against the 160 kD subunit of Neurofilament (anti-NF; AMAC Inc.) and a rabbit polyclonal antibody raised against the murine Engrailed (En) proteins were used to control for the specificity of the immunohistochemistry.

20 All observations reported here have been derived from a number of independent experiments all of which gave similar results. The total number of anti-Nuk stained embryos observed at each stage was from 40 to over 100.

Embryos used in this study were obtained from natural matings of CD1 mice and at the required stages of development were dissected and membranes were removed in ice cold 0.1 M phosphate buffer (pH 7.4). Embryos were then fixed with occasional mixing for 2 h on ice in fresh 4% paraformaldehyde in 0.1 M phosphate buffer. In some instances 0.2 to 1% glutaraldehyde was also added to the fixative. The fixative was washed out overnight in 0.1 M phosphate buffer at 4°C prior to gently dehydrating the embryos in a methanol/phosphate buffered saline (PBS) series (15%/85%; 30/70; 50/50; 75/25% H₂O; 100% methanol) on ice for 30 minutes each step. Once in 100% methanol the embryos can be stored at -20°C for at least 2

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months. Prior to rehydration and further manipulation, the embryos were transferred into 80% methanol/20% H₂O₂ for 4-6 h at RT to bleach embryos and inactivate endogenous peroxidases. After washing in 100% methanol, the embryos were rehydrated on ice for 30 minutes each with occasional mixing in

5 75% methanol/25% H₂O, 50% methanol/50% PBS, 30% methanol/70% PBS, 15% methanol/85% PBS, and 100% PBS + 0.01% Triton X-100. The embryos were then gently mixed on a Nutator at RT 2 x 1 h in PBSMT (2% Dry Milk Powder, 0.01% Triton X-100 in PBS). At this step a small number of embryos were placed at 4°C in PBSMT for preabsorbtion of the biotinylated secondary

10 antibodies as described by the manufacturer (Vector Laboratories). The embryos were then blocked overnight at 4°C on Nutator using the Vectastain ABC Elite and blocking kits in PBSMT+3% normal goat serum (NGS) + 10% avidin blocking reagent. The avidin was then washed out in PBSMT 4 x 1 hour at 4°C followed by 2 x 1 hour at RT. The blocked embryos were then

15 incubated on a Nutator for at least 16 h at 4°C with affinity-purified anti-Nuk antibodies (0.5 to 1.0 mg/ml) in PBSMT containing 3% normal goat serum and 10% biotin blocking agent. Unbound primary antibodies and biotin were washed out in PBSMT 4 x 1 hour at 4°C followed by 2 x 1 hour at RT. The preabsorbed biotinylated secondary antibodies were then added to the embryos

20 and incubation was carried out overnight at 4°C on a Nutator. The secondary antibodies were washed out as described above and the Vectastain ABC elite avidin-biotin-HRP reagent in PBSMT+3% NGS was added and allowed to incubate overnight at 4°C on a Nutator. The ABC elite avidin-biotin-HRP reagent was washed out as described above ending with a final wash in PBT

25 (0.2% BSA, 0.01% Triton X-100 in PBS) at RT. For HRP detection, embryos were incubated in 0.3 mg/ml diaminobenzidine (DAB) in PBT at RT for at least 20 minutes. H₂O₂ was added to 0.03% and the embryos were incubated at RT under a dissecting microscope until color density was sufficient, usually about 1-10 minutes. The color of HRP-DAB reaction product can be changed from an

30 orange to dark purple color by the addition of NiCl₂ to 0.5%. After staining, embryos were washed in PBT and dehydrated through a ethanol/PBS series: 30/70, 50/50, 80/20, 100% ethanol for 30 minutes each. For light microscopy,

embryos were cleared in benzyl alcohol:benzyl benzoate (1:2). Photography was carried out with either a Wild M10 macroscope, or a Leitz DMRXE compound microscope using Kodak EPY 64 Tungston slide film or Kodak Ektar 100 print film. Whole-mount mRNA in situ were performed as described in Canton & Rossant, (1992) Development: 116, 357-368.

Paraffin section immunohistochemistry

For the immunohistochemical staining of sectioned embryos, dissected animals were fixed with occasional mixing for 2 h on ice in 4% paraformaldehyde + 1% glutaraldehyde in 0.1 M phosphate buffer. Embryos were then washed in phosphate buffer and dehydrated on ice for 30 minutes each with occasional mixing in 25% ethanol/75% PBS, 50% ethanol/50% PBS, 70% ethanol/30% H₂O prior to storage at 4°C. Before embedding in paraffin, embryos were completely dehydrated in 85% ethanol/15% H₂O and then 100% EtOH. The ethanol was replaced with multiple changes of xylene at RT, and the embryos were then equilibrated to 60°C. The xylene was exchanged with many changes of melted paraffin for 2 to 4 hours at 60°C prior to embedding. Embryos were sectioned at 4 to 6 µm and placed on slides freshly subbed in 1% aminopropyltriethoxysilane. Sections were placed on a slide dryer for 1 hour, allowed to dry overnight at RT, and then partially melted at 55°C for 20 min.

The antibody staining was performed exactly as described in the Vectastain ABC Elite instructions. Anti-Nuk antibodies were used at 4 to 8 µg/ml. For Nuk+peptide control 8 µg/ml of trpE-Nuk fusion protein was added to the anti-Nuk antibodies and preincubated for 1 hour before adding to the sections. Anti-NF monoclonal antibodies were used at 4 µg/ml. In some instances, the HRP reaction was carried out in DAB only. These sections were counterstained in hematoxylin prior to mounting with coverslips. In other sections, the HRP reaction was carried out in DAB+NiCl₂ to produce a darker higher contrast product for black and white photography. These sections were not counterstained prior to mounting. Photography was performed on a Leitz DMRXE microscope using Kodak EPY 64 tungsten color slide film or Kodak Technical Pan black and white print film.

Transmission electron microscopy

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Since the HRP-DAB reaction product produced in the immunohistochemical staining is electron dense, the subcellular localization of Nuk protein using transmission electron microscopy (EM) of sectioned anti-Nuk protein whole mount stained embryos was performed. Similar
5 ultrastructural immunoperoxidase localization studies of the mammalian embryonic central nervous system has been performed using antibodies directed against the glial fibrillary acid protein (Levitt *et al.*, 1981) or stains specific for the extracellular matrix (Nakanishi, 1983). Embryos were fixed in 4% paraformaldehyde + 1% glutaraldehyde and then immunoreacted as
10 whole-mounts with anti-Nuk antibodies as described above. The stained embryos were postfixed in OsO₄, dehydrated, and embedded in Spurr resin. Ultrathin sections were observed and photographed on a Philips EM430 transmission electron microscope. To render the HRP reaction product more visible, counterstaining of the ultrathin sections with uranyl acetate and lead
15 citrate was omitted. To control for fixing conditions and morphology, similar staged and fixed embryos were postfixed in OsO₄ and prepared immediately for EM analysis. Other than a loss of lipids from membranes due to extensive washing, the morphology of the immunoreacted samples was comparable to the controls.

20 EXAMPLE 1

Cloning and chromosomal location of Nuk

The initial *Nuk* cDNA clone, designated λ Q1, was isolated from an unamplified λ gt11 expression library constructed from a mouse erythroleukemia cell line by screening with anti-phosphotyrosine antibodies
25 (Ben-David *et al.*, *EMBO* 10:317-325, 1991). Sequence analysis of this clone indicated it was a partial cDNA whose expected translation product was closely related to members of the Eph family of receptor-like tyrosine kinases. Standard cDNA screening of a 12.5 day mouse embryo cDNA library and rapid amplification of cDNA ends (RACE) from 9.5 day embryo mRNA was used to
30 clone the remainder of the *Nuk* coding region (except for the A of the presumptive ATG initiation codon) following the methods described above.

Figure 2 shows that the translation of the combined *Nuk* RACE and cDNA sequences revealed a single open reading frame of 994 amino acids containing both a hydrophobic signal peptide and transmembrane domain (long underlines, Figure 2). All the hallmarks of a receptor tyrosine kinase of the Eph family are found in Nuk protein, including 20 cysteine residues whose position is conserved in the extracellular domain of Eph family members (bold type, Figure 2), an immunoglobulin-like domain near the amino terminus (Ig-like), and two fibronectin type III repeats (FN III; between Nuk amino acids residues 330-420 and 444-534). The Ig-like domain of Nuk contains specific residues (Cys⁷⁰, Trp⁸⁰, Cys¹¹⁵) known to be conserved in the Ig superfamily (Williams and Barclay, *Ann. Rev. Immunol.* 6:381-405, 1988). When compared to the Ig-like domains found in other receptor tyrosine kinases (O'Bryan *et al.*, *Mol. Cell. Biol.* 11:5016, 1991), the Nuk Ig-like domain was found to contain a number of conserved residues (overlines, Figure 2). A repeat involving the Nuk Ig-like domain and residues 239 to 268 is apparent (underlines, Figure 2). Although significantly shorter than a normal Ig domain, residues involved in the Nuk-specific repeat correspond to conserved residues often found in Ig-like domains (residues that are both overlined and underlined in Figure 2). Following the transmembrane domain, the Nuk cytoplasmic region contains a tyrosine kinase catalytic domain (brackets, Figure 2) and a carboxy-terminal tail of 106 residues.

The cartoon in Figure 2 shows the location of the various domains. The carboxy-terminal region used to raise the anti-Nuk antibodies is indicated in Figure 2.

Following a 26 amino acid hydrophobic signal peptide, the Nuk protein extracellular domain is composed of an Ig-like domain and two FN III repeats. The Nuk protein extracellular domain also contains 20 cysteines whose position is conserved in the Eph family (Lhotak *et al.*, *Mol. Cell. Biol.* 11:2496-2502, 1991). A hydrophobic transmembrane domain divides the Nuk protein into approximately two halves, a 548 amino acid extracellular region and a 419 amino acid cytoplasmic region which contains a tyrosine kinase catalytic domain.

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The Nuk protein sequence was compared to other known members of the *Eph* family. Nuk was found to be most highly related to the full length amino acid sequence of chicken Cek5 (96% identity; Pasquale, *Cell Regulation* 2:523-534, 1991) and to short PCR products of mRNA from rats (*Tyro 5*; Lai and Lemke, *Neuron* 6:691-704, 1991) and humans (*Erk*; Chan and Watt, *Oncogene* 6:1057-1061 1991). The close identity between *Nuk* and *Cek5* suggest they represent the mammalian and avian orthologs of the same progenitor gene. The absence of full length cDNAs for *Tyro 5* and *Erk* precludes the determination of whether these sequences correspond to the same or a closely related but different gene.

The chromosomal location of *Nuk* was determined by probing for restriction fragment length polymorphisms (RFLPs) in the DNA of a number of recombinant inbred mouse strains derived from matings between AKR/J and DBA/2J mice (B.A. Taylor, personal communication). The *Nuk* locus mapped to the distal end of mouse chromosome 4 near the *ahd-1* mutation.

EXAMPLE 2

Nuk tyrosine kinase activity in mouse embryos

To investigate the biological function of Nuk protein, antibodies were raised against a bacterial fusion protein containing the C-terminal 94 amino acids of Nuk (trpE-Nuk). Anti-Nuk protein antibodies were affinity-purified by binding to a bacterial GST-Nuk fusion protein. The specificity of the anti-Nuk protein antibodies was assayed by immunoblotting of bacterial lysates expressing either β gal-Nuk or β gal-Elk carboxy-terminal domains. This experiment demonstrated that the anti-Nuk protein antibodies recognized only the β -gal-Nuk fusion protein. To confirm this result with mammalian protein extracts, Elk protein was immunoprecipitated from a rat brain protein lysate with anti-Elk antibodies. Western blot analysis of the immune-complexes verified that anti-Elk, but not anti-Nuk antibodies could recognize Elk. These experiments demonstrated that the affinity-purified anti-Nuk protein antibodies do not cross-react with the related Elk protein.

To assay for Nuk tyrosine kinase activity *in vitro*, Nuk protein was

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immunoprecipitated from 10.5, 12.5, and 14.5 day mouse embryo protein lysates and then incubated in the presence of [γ^{32} P]ATP. The expression and tyrosine kinase activity of Nuk in the embryos is shown in Figure 3.

5 Anti-Nuk protein antibodies immunoprecipitated a protein-tyrosine kinase of 135 kD, as shown in Figure 3A. A highly phosphorylated protein with a relative mobility of 135 kD was detected in protein extracts of 10.5 day (lane 3, Figure 3A), 12.5 day (lane 4, Figure 3A), and 14.5 day (lane 5, Figure 3A) embryos. The mobility of this protein is consistent with that reported for other Eph family members including Eck, Elk, and Cek5 (Lindberg *et al.*, *Mol. Cell. Biol.* 10:6316-6324, 1990; Lhotak *et al.*, *Mol. Cell. Biol.* 11:2496-2502, 1991; Pasquale *et al.*, *J. Neurosci.* 12:3956-3967, 1992). No kinase signal was observed from 10.5 day embryo lysates when preimmune serum was used (lane 1, Figure 3A) or if the anti-Nuk antibodies were initially preincubated with a trpE-Nuk fusion protein (lane 2, Figure 3A). To enrich for phosphotyrosine, 15 the gel was treated with KOH as described previously herein.

Similar anti-Nuk protein immunoprecipitates were also subjected to Western blot analysis with anti-Nuk antibodies, as shown in Figure 3B. Immunoblotting identified Nuk protein as a 135 kD protein, (Figure 3B). The anti-Nuk protein antibodies detected Nuk protein as a 135 kD protein in 10.5 day (lane 3, Figure 3B), 12.5 day (lane 4, Figure 3B), and 14.5 day (lane 5, Figure 3B) embryos. No signal was detected if the 10.5 day embryo lysate was immunoprecipitated with preimmune serum (lane 1, Figure 3B), or with anti-Nuk antibodies pre-incubated with a trpE-Nuk fusion protein (lane 2, Figure 3B). The abundant low molecular weight signals are due to binding by 25 the goat anti-rabbit secondary antibodies to the anti-Nuk protein antibodies and the IgG in the preimmune serum used in the immunoprecipitation. In agreement with the *in vitro* kinase studies, the Western blot revealed highest levels of the 135 kd Nuk protein in 10.5 day mouse embryos (Figure 3B, lane 3).

30 EXAMPLE 3

Segmental expression of Nuk

The biochemical data presented above and Northern mRNA analysis

indicated *Nuk* is highly expressed in 9.5 to 10.5 day mouse embryos. To investigate this expression in detail, *Nuk* protein was visualized in 7.5 to 13 day mouse embryos *in situ* using the anti-*Nuk* protein antibodies in whole-mount and paraffin section immuno-histochemical techniques. To
5 confirm the protein studies, *Nuk* mRNA *in situ* on whole-mounts (Conlon and Rossant, *Development* 116:357-368, 1992) and frozen sections were also performed with similar staged embryos.

Nuk protein localization in whole-mount and paraffin sections of 7.5 to 10.5 day mouse embryos is shown in Figure 4, comprising whole-mount
10 preparations showing *Nuk* mRNA (Figure 4A) and protein (Figures 4B to 4E) at early postimplantation stages of embryonic development. Unless otherwise stated, in Figure 4, dorsal is *left* and anterior is *up*. Figure 4A represents whole-mount mRNA studies which detected *Nuk* transcripts enriched in the neural ectoderm of an embryo at 7.5 days development (note dorsal surface is
15 *up*). By the 6 somite stage (8 days) *Nuk* protein is detected as dark orange horseradishperoxidase (HRP) staining as shown in Figure 4B. This is most apparent in the neural groove where relatively high levels are observed in specific hindbrain rhombomeres and in the midbrain region. The arrowhead in Figure 4B points to rhombomeres r2, r3 that express high levels of *Nuk*
20 protein. The sinus venosis of the developing circulatory system also stains positive for *Nuk*. Figure 4C shows an embryo at 12 somite stage (8.75 days) and reveals elevated levels of *Nuk* protein in specific regions of the anterior neural tube. The most anterior structures immunoreactive for *Nuk* include the ventral diencephalon followed by the ventral mesencephalon/midbrain.
25 Rhombomeres r2, r3, and r5 of the hindbrain are also immunoreactive for *Nuk* protein. The arrow in Figure 4C points to rhombomere 5. The expression of *Nuk* was confirmed by performing mRNA *in situ* on similar staged embryos.

Figure 4D shows 24 somite (9.5 day) stage embryos immunoreacted
30 with either anti-*Nuk* protein antibodies (left) or, as a control, anti-*Nuk* protein antibodies that were preincubated with a trpE-*Nuk* fusion protein (right). *Nuk* protein is most abundant in the ventral midbrain, diencephalon, and optic

stalk. Along the spinal cord, high levels of Nuk protein is detected at the dorsal surface of the neural tube (see Figure 5 for more detail). Note the absence of specific staining in the control embryo. Figure 4E shows whole-mount immunolocalization of Nuk protein in the developing brain of a 9.5 day embryo in greater detail. Nuk protein is most highly expressed in the ventral midbrain encompassing the flexure region. Nuk protein is also detected in the ventral diencephalon, optic chiasm, optic stalk, retinal cells (out of focus), and basal telencephalon. At this stage, Nuk protein is still detected in the hindbrain and is localized to the floorplate (arrow in Figure 4E points to ventral region of rhombomere 2). Note the small patch of Nuk protein now detectable in the lateral region of rhombomere 4 just anterior of the otic vesicle.

Figures 4F to 4J show immunohistochemical detection of Nuk protein in paraffin sections of 9.5 and 10.5 day mouse embryos. Figures 4F and 4G show serial transverse sections of a 9.5 day embryo immunoreacted with anti-Nuk antibodies which detect Nuk protein (brown stain) in the ventral midbrain. Nuk protein is localized to all layers of the neural tube including the proliferative ventricular zone, the internal mantel layer, and the outer marginal layer which is adjacent to the surrounding mesenchyme. Note Nuk protein is detected only in the ventral aspect of the midbrain and its limit of expression marks a specific morphological bulge/constriction of the neural tube that separates ventral from dorsal components (arrowhead).

Figure 4H shows a sagittal section along the midline of a 10.5 day embryo showing Nuk protein (dark stain) concentrated in ventral regions of the midbrain and in the optic chiasm (asterisk) and footplate of the hindbrain (thick arrow). The orientation of this embryo is opposite to the one shown in Figure 4E. Note the appearance of a morphological constriction (arrowhead) which separates the midbrain from the diencephalon. Since this is a section along the midline, the infundibulum or ventral most region of the diencephalon is exposed between the midbrain and optic chiasm. This region of the diencephalon does not express Nuk. Figures 4I and 4J show adjacent transverse sections of a 10.5 day embryo immunoreacted with either anti-Nuk

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protein antibodies (4I) or anti-Nuk antibodies preincubated with a trpE-Nuk peptide (4J), and illustrate the specificity of the immunohistochemistry. Staining of Nuk protein at 10.5 days persists in the ventral midbrain and is also detected at lower levels in the floorplate of the hindbrain (arrowhead).

5 The scale bars shown in Figure 4 represent the following measurements: (4A), 100 μ m; (4B and 4C), 120 μ m; (4E, 4F, 4G, 4I, 4J), 150 μ m; (3H), 300 μ m.

 As described above, by the six somite stage (8 days) high levels of Nuk protein are detected in the neural ectoderm prior to fusion of the dorsal
10 edges of the neural plate (Figure 4B). Highest levels of Nuk immunoreactivity are detected in the presumptive midbrain and in specific rhombomere segments of the hindbrain. Nuk protein is also detected in the sinus venosus of the developing circulatory system. By the 12 somite stage (8.75 days) the dorsal edges of the neural plate have fused forming the neural tube. High levels of
15 Nuk protein are localized to specific compartments of the neural tube including hindbrain rhombomeres r2, r3, and r5, the ventral midbrain (mesencephalon) encompassing the flexure, and the ventral diencephalon (Figure 4C).

 By 9.5 to 10.5 days of development, *Nuk* expression intensifies in
20 specific ventral regions of the developing brain structures (Figures 4D and 4E). In the hindbrain at this stage, Nuk protein becomes concentrated along the floor plate. When anti-Nuk protein antibodies are used to stain transverse sections at the midbrain of 9.5 day (Figures 4F and 4G) and 10.5 day (Figure 4I) embryos, it is apparent that Nuk protein is restricted to the morphological
25 bulge corresponding to the ventral/basal plate of the mesencephalon. Preincubating the anti-Nuk protein antibodies with a Nuk peptide abolished the signal, demonstrating the specificity of the antibody in both whole-mount and paraffin section immunohisto-chemistry (Figure 4D and 4J). Staining of additional transverse sections determined that Nuk protein in the
30 diencephalon of 9 to 10.5 day embryos is also restricted to ventral structures, including the hypothalamus, thalamus, and the optic stalk. Very high levels of Nuk protein were also detected in cells of the primitive optic chiasm at day

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10.5. Sagittal sections confirmed that high levels of *Nuk* mRNA and protein (Figure 4H) are detected specifically in ventral cells of the developing brain. Consistent with the *in vitro* kinase and Western analysis, anti-Nuk immunoreactivity in 11.5 to 13.5 day embryos decreases significantly in these
5 brain structures as discussed below.

EXAMPLE 4

Subcellular localization of Nuk

The immunohistochemical reaction product observed for the anti-Nuk antibodies often appears as if the Nuk protein is localized to specific
10 surfaces of the plasma membrane. One clear example of this is at the dorsal surface of the posterior neural tube which contains elevated amounts of the Nuk protein as early as 9.5 days of development (see Figure 4D). The subcellular localization of Nuk protein is shown in more detail in Figure 5.

Figures 5A and 5B are adjacent transverse sections of an 11.5 day
15 embryo at the level of the caudal/posterior spinal cord immunoreacted with either anti-Nuk antibodies (5A) or anti-Nuk antibodies preincubated with a trpE-Nuk peptide (5B). Nuk protein is concentrated in the dorsal region of the neural tube along the basement membrane (arrow). This localization of Nuk protein is observed as early as 9.5 days of development in whole-mount
20 preparations (see Figure 4D). Antibodies to Neurofilament (anti-NF) and Engrailed (anti-En) proteins did not stain the basement membrane demonstrating the specificity of the anti-Nuk antibodies.

Figures 5C and 5D show transmission electron microscopy (EM)
immunolocalization of Nuk protein in ventral midbrain cells of a 9.5 day
25 embryo. Immunoperoxidase anti-Nuk stained whole-mount embryos were ultrathin sectioned and observed under EM. Figure 5C shows that, at the proliferative ventricular zone (VZ), Nuk-positive signals are concentrated at sites of cell-cell contact (arrows). Notice that Nuk protein immunostaining is often localized on the membranes of both cells that are making contact. At this
30 stage of development there is no obvious ultrastructural differences between neuronal and glial cell types. Therefore, it cannot be distinguished if Nuk protein is associated with neurons, glial, or both cell types. Note the condensed

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chromatin evident in the nucleus of a cell that is in late prophase (asterisk).

Figure 5D shows that cells within the postmitotic mantle region of the neural tube also exhibit Nuk protein localization at sites of cell-cell contact. The photomicrograph shows a site of contact between two cells labelled
5 strongly for Nuk protein (filled arrow). Note that other sites of cell-cell contact do not contain detectable amounts of Nuk protein (open arrows).

The scale bars in Figure 5 represent the following measurements: (A and B) 100 μ m; (C), 2 μ m; (D), 1 μ m.

Transverse sections of 11.5 day embryos shows in greater detail that
10 Nuk protein is concentrated where the dorsal-most membrane of the neural tube make contact with the surrounding mesenchyme/neural crest cells (Figures 5A and B). Lateral and ventral regions of the neural tube do not exhibit this localized Nuk protein.

Since the HRP-DAB reaction product produced in the
15 immunohistochemical staining is electron dense, the subcellular localization of Nuk protein was investigated using transmission electron microscopy (EM), following the methods described above. The ventral midbrain of 9.5 day embryos was chosen for EM analysis because of its intense Nuk immunoreactivity. Figure 5C shows a section of the midbrain at the
20 mitotically active ventricular zone. Nuk immuno-reactivity associated with these cells is usually localized to the plasma membranes at specific sites of cell-cell contact. As shown in Figure 5D, Nuk immunoreactivity was not found to be localized to all sites of cell-cell contact. In addition, small punctate deposits throughout the ventral midbrain were immunoreactive for Nuk
25 protein. These structures appear to be plasma membrane material lost from post-mitotic cells during their radial migration from the ventricular zone.

EXAMPLE 5

Nuk localization in pioneer cranial PNS axons and an early CNS axon tract and in spinal axons

30 Axonogenesis in mouse embryos commences at approximately 10 days of development when neurons associated with both the central and peripheral nervous systems extend axon projections toward their targets.

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Anti-Nuk antibody staining of 10 to 12 day embryos revealed that Nuk protein is highly concentrated within most if not all early axonal projections of the PNS and to at least one early pathway of the CNS.

5 An example of Nuk protein localization in a specific cranial nerve, the oculomotor nerve (III), is shown in Figure 6. Figure 6A is a photomicrograph of the head of an 11 day anti-Nuk whole-mount immunostained embryo with one of the pair of oculomotor nerve fibers in focus showing strong labelling of Nuk protein (filled arrow). A clearer view of Nuk protein staining in the oculomotor axons fibers can be obtained when the
10 whole-mount staged embryo was filleted down the midline to minimize tissue thickness (Figure 6B). This view shows that the Nuk protein positive oculomotor III axons exit the neural tube from the ventral aspect of the midbrain. An anti-Nuk stained frontal section shows that these axons originate from the Nuk-expressing cells in the ventral midbrain, at a region
15 that is consistent with the position of the oculomotor nuclei (Figure 6E). Anti-Nuk antibodies also label an early pathway of the CNS (open arrow in Figure 6).

Figures 6C to 6F show paraffin section immunohistochemistry of 10.5 to 11 day mouse embryos. Adjacent transverse sections bisecting the pair
20 of oculomotor nerve fibers were immunostained with either anti-Nuk (6C) or anti-Neurofilament (6D) antibodies. The arrows in both Figures 6C and 6D point to the darkly stained axon bundles of the two oculomotor nerves. To verify the specificity of the immunohistochemistry, additional control experiments were performed in which the anti-Nuk antibodies were either
25 omitted, preincubated with a trpE-Nuk peptide, or substituted with antibodies directed against the Engrailed (anti-En) nuclear homeodomain proteins. Figure 6E is a frontal section immunostained with anti-Nuk antibodies which label the ventral midbrain and oculomotor axon fibers as they exit the neural tube (filled arrow) and extend (open arrow) towards their target tissue, the
30 pre-optic muscle mass.

Figure 6F shows a sagittal section immunostained with anti-Nuk antibodies which label the oculomotor axon fasciculus as it enters the pre-optic

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muscle mass (open arrow). This section also shows the expression of Nuk protein in the developing retinal cells (r). The orientation of this section is the same as Figure 4H.

5 The scale bar for Figures 6 (A) is 180 μm ; (B) 120 μm ; and (C-F) 100 μm .

To confirm that the observed Nuk protein localization is in axons, adjacent transverse sections that bisect the oculomotor nerve fibers of a 10.5 day embryo were immunoreacted with either anti-Nuk (Figure 6C) or neuron-specific anti-Neurofilament antibodies (anti-NF; Figure 6D). Both
10 antibodies labelled the same structure demonstrating the axon-specific localization of Nuk protein. The main target tissue of oculomotor axons is the premuscle mass of cells adjacent to the developing retina, whose function in the adult is to control certain eye movements. An anti-Nuk stained sagittal section labelled the oculomotor axon fascicule at the point of entry into the
15 premuscle mass (Figure 6F). This section also shows the high level of Nuk protein associated with the developing retinal cells.

Other cranial PNS axons contain high levels of Nuk protein early during their elongation. Nuk protein is detected in the trigeminal (V) and facial (VII) nerve fibers in 10.5 day embryos (Figures. 7A to 7C). The vagus (X)
20 parasympathetic autonomic nerve, and the accessory (XI) and hypoglossal (XII) somatomotor nerves also contain localized Nuk protein (Figures. 7D and 7E). As observed by the anti-Nuk labelling, these fibers enter a common region, the cardiac/pulmonary plexus, where they then elongate to their targets such as the cardiac muscle and other visceral organs (vagus) or the upper torso
25 (accessory) and tongue (hypoglossal). Nuk protein localization in these cranial axons is very transient and is not detected after 12.5 days development.

The embryo in Figure 6A and B also exhibits specific anti-Nuk labelling in the developing CNS of a connection between the telencephalon and the midbrain. Information describing the naming and position of early
30 tracts in the developing mammalian forebrain is sparse. The location of Nuk immunoreactivity is consistent with the location of axonal projections originating from the ventral midbrain tegmentum such as those of the red

nuclei or the reticular activating system (Carpenter, 1985, Core Text on Neuroanatomy, Baltimore: Williams & Wilkins). Alternatively, this pathway may correspond to the telencephalic/supraoptic tract described in zebra (Chitnis and Kuwanda, J. Neurosci. 10, 1892-1905, 1990; Wilson et al., (1990) Development, 108, 121-143, 1990). Closer definition of the origin and termination sites as well double labelling with other antibody probes should help determine the identity of this Nuk-positive tract.

EXAMPLE 6

Nuk Localization in Spinal Axons

Figures 7A to 7E show Nuk protein localization in cranial nerves of 10.5 to 12 day mouse embryos. Figure 7A is a 10.5 day whole-mount embryo immunostained with anti-Nuk antibodies which label the trigeminal nerve V and facial nerves VII. Figures 7B and 7C show transverse sections of a 10.5 day embryo showing Nuk-positive trigeminal nerve V axon fascicules labelled with the anti-Nuk antibodies. Note that Nuk protein is also present throughout the caudal hindbrain region of the neural tube. Figures 7D and E are 11.5 day anti-Nuk whole-mounts showing localization of Nuk protein in the vagus nerve (X) associated fibers as they pathfind to their target visceral organs (curved open arrows). Other cranial axons including the accessory (XI) and hypoglossal (XII) nerve fibers are immunoreactive for Nuk protein. These cranial nerves initially extend to the plexus region (open arrow in D) before they pathfind to the heart and other target tissues.

Figures 7F to 7L show Nuk protein localization in spinal nerves of 10 to 12 day embryos. Figure 7F is a whole-mount 10 day embryo showing Nuk protein concentrated within the earliest spinal nerve fibers exiting the neural tube (arrows). Figure 7G shows a slightly later stage embryo from that shown in Figure 7F. Notice that the nerve fibers exiting the neural tube have thickened due to fasciculation of additional axons. The insert is a close-up of a single spinal nerve showing Nuk protein is localized throughout its length and can be observed at the leading tips of the growth cones. Figures 7H and 7I show transverse sections bisecting the rostral spinal cord of an 11 day embryo

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demonstrating that the darkly stained Nuk-positive fibers shown in Figures 7F and 7G are ventral motor axons (open arrows). At this stage, the accessory nerve also stains positive for Nuk protein as evident by the strong labelling of the axon fibers (filled arrows). The uniform expression of Nuk throughout the spinal region of the neural tube is apparent in these sections.

Figures 7J and 7K show both sides of an 11 day whole-mount embryo demonstrating that Nuk protein is localized within the spinal motor nerves as they elongate to the plexus regions (open arrows in Figure 7J). Between the motor fibers, the appearance of Nuk-positive DRG cell bodies and axons can be observed in this embryo (filled arrows in Figure 7K). Note that the Nuk-positive DRG axons are more apparent in the posterior/caudal segments of the spinal cord. Figure 7L shows a close-up of a 12 day whole-mount embryo immunostained with anti-Nuk antibodies which label the nerve fibers of the parasympathetic chain ganglion. Note the ganglia form a chain of interganglionic axonal connections with each other and that each ganglion unit forms connections with two segments of the neural tube.

Scale bars in Figures 7B, 7C, 7H, and 7I represent 200 μ m.

Immunostained whole-mount 10-10.5 day embryos revealed high levels of Nuk protein in the earliest spinal motor axons as they exit the neural tube (Figures 7F and G). The close-up photomicrograph in Figure 7G shows that Nuk protein is localized throughout the length of the axons including the growth cones. Transverse sections immunostained with anti-Nuk antibodies confirmed that Nuk protein is highly localized to the ventral motor axons and the axons of the DRG (Figures 7H and I). Note detectable levels of Nuk protein throughout the spinal cord. By 11 days of development (Figures 7J and K) the Nuk-positive nerve fibers have elongated and thickened considerably due to the fasciculation of additional axons along the initial axons which, by this time, have reached the plexus regions (Tosney and Landmesser, *J. Neurosci.* 4:2518-2527, 1984; Tosney and Landmesser, *Dev. Biol.* 109:193-214 1985a; Tosney and Landmesser, *J. Neurosci.* 5:2336-2344 1985b; Landmesser and Swain, *Neuron* 8:291-305, 1992). Nuk protein in the rostral DRG cell bodies and their fibers connecting to the neural tube is also detected (Figures 7J and 7K). By 12

days of development only low levels of Nuk immunoreactivity are detected in the motor and DRG axons. At this stage, Nuk protein is detected in axon fibers of the sympathetic chain ganglion (Figure 7L) and nerve fibers surrounding the heart and diaphragm regions.

5 **EXAMPLE 7**

Nuk protein early in ear development

Very high levels of Nuk protein were found in specific structures of the developing ear and associated vestibulocochlear (VIII) ganglion in 10.5 to 12.5 day embryos as is shown in Figure 8.

10 As shown in Figure 8, anti-Nuk immunoreacted whole-mount 10.5 day embryo detects Nuk protein localization (arrow) at the dorsal region of the otic vesicle (ov) surrounding the budding endolymphatic duct (Figure 8A). Branchial arches 1 and 2 are indicated. By 11.5 days of development the endolymphatic duct has elongated approximately 200µm dorsally (Figure 8B).
15 Nuk protein is observed to envelope this structure (arrow). Nuk protein localization within cranial nerve VII as it enters the second branchial arch can also be seen in Figure 8B. Figure 8C shows a slightly different focal plane from (B) showing that the vestibulocochlear sensory fibers connecting to the developing ear stain positive for Nuk protein (arrow). Nuk protein associated
20 with the developing eye is also shown in Figure 8C. Transverse section of an 11.5 day embryo detects high levels of Nuk protein localized to the basement membrane of the endolymphatic duct cells where they contact the surrounding mesenchymal/neural crest cells (open arrow, Figure 8D). Nuk protein associated with the vestibulocochlear ganglion is also visible in this
25 section (filled arrow).

The scale bars in Figure 8 represent the following: (8A), 100 µm; (8B and 8C), 200 µm; (8D), 100 µm.

At 10.5 days Nuk immunostaining associated with the ear is first observed at the dorsal region of the otic vesicle surrounding the initial bulge
30 of the endolymphatic duct (Figure 8A). By 11.5 days Nuk immunoreactivity encapsulates the endolymphatic duct during its dorsal elongation (Figure 8B). A different focal plane of the same embryo shows Nuk is also localized to the

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vestibulocochlear ganglion and its axon fibers connecting to the developing ear (Figure 8C). Transverse sections detected Nuk protein specifically localized to the basement membrane of the endolymphatic duct cells (Figure 8D). By 12.5 days of development only low levels of Nuk were detected in the endolymphatic duct and the acoustic ganglion.

EXAMPLE 8

Generation of Loss of Function *Nuk* mutant

A loss of function mutation in *Nuk*, designated *Nuk*¹ was generated in embryonic stem cells, and germline transmission of the null allele was obtained as described in more detail below.

A null allele was generated in mouse embryonic stem cells generally following the methodology described in Capecchi M.R., Science 244:1288-1292, 1989. The null mutation was obtained by deletion of exon 2, corresponding to codons 29 to 50, as shown in Figure 9. To obtain germ line transmission of the mutation *Nuk*^{+/-} embryonic stem cell lines (ES) were aggregated with 8 cell embryos *in vitro* and the resulting blastocysts were transferred into recipient females. Upon birth, animals chimeric for ES and embryonic stem cells were recovered by scoring for eye pigment and coat colour. Breeding of these "aggregation chimeras" confirmed that the germ line of at least one founder mouse is derived completely from the ES cells. Adult mice homozygous for the mutation did not express Nuk protein.

EXAMPLE 9

Generation of a *Nuk-lac Z* fusion chimeric receptor mutant

A targeted mutation, designated *Nuk*² was generated in the *Nuk* gene as shown in Figure 10. A *pPNT-LOX-Nuk*² gene trap vector was used to delete the GXGXXG ATP binding region of the kinase domain (amino acids 623-707, SEQ ID NO:2 and Figure 2) to create a *Nuk-lac Z* fusion receptor in ES cells. Chimeric animals were prepared as described above, by aggregating the ES cells with 8 cell CD1 embryos.

Animals generated with the *Nuk*² mutation provided *Nuk* expressing cells staining for β -galactosidase activity, providing a convenient marker for Nuk-positive cells in both heterozygous and homozygous

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backgrounds. The *Nuk²* mutation led to the expression of a Nuk-lac Z fusion protein in mouse heterozygous embryos, detected by a blue/green colour as shown in Figures 11 and 12.

Figure 11A shows an embryo at the six somite stage (8.25 days development) expressing the *Nuk²* mutation in the brain and developing heart. Figure 11B shows that at the 14 somite stage (8.75 days development) expression continues in the hindbrain rhombomeres, the midbrain and diencephalon and persists in the heart. Figure 12 shows *Nuk²* expression in a 10.5 day old embryo. Figure 12A illustrates the very high levels of expression in the ventral midbrain, diencephalon and retinal cells (which are out of focus in the photomicrograph). Figure 12B illustrates expression in the brain and spinal cord.

EXAMPLE 10

Autophosphorylation of Nuk protein by Elk-ligand

Fusion proteins consisting of the extracellular domain of Elk-ligand (Davis, S., et al. Science Vol. 266, Nov. 4, 1994, p.816) linked to the Fc portion of human immunoglobulin G1 were made in COS cells S/N following the methods outlined in Davis et al, 1994, supra. The expressed ligand was aggregated with anti-human Fc antibody and incubated with COS cells for 5 minutes, 15 minutes, 30 minutes or 1 hour. Following incubation, the COS cells were lysed and the lysate was immunoprecipitated with anti-Nuk antibodies. The immunoprecipitates were subjected to Western blotting analysis with anti-phosphotyrosine antibodies. Figure 13 shows the autophosphorylation of Nuk on tyrosine from 15 minutes to one hour following Elk-ligand stimulation. Therefore, Nuk autophosphorylation is induced by stimulation with the Elk-ligand.

EXAMPLE 11

Nuk expression in Cell Lines

Various cell lines were screened for expression of Nuk protein. Cells were homogenized and the homogenate was incubated with anti-Nuk antibody and the immunoprecipitates were collected by centrifugation. The washed immunoprecipitates were separated by SDS-polyacrylamide gel electrophoresis

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and subjected to Western blotting with anti-Nuk antibodies or anti-phosphotyrosine antibodies. As shown in Figures 14 and in Table 1, COS cells expressed high levels of Nuk protein.

5

TABLE 1

	<u>MG87</u>	<u>COS</u>	<u>Rat2</u>	<u>PC12*</u>
Nuk protein	+	++++	+	+

(*NB/ can't stimulate with elk-ligand)

10

EXAMPLE 12**GST-fusion mixing experiments with Nuk in vitro phosphorylation**

15

GST-fusion proteins containing SH2 domains of p85N, p85C, PLC γ N, PLC γ C, GAPN, Src, Crk, Abl, and Grb2 were made in E. coli. Nuk protein was immunoprecipitated from lysates of COS cells using Nuk antisera, and phosphorylated by incubation with ATP. Bacterial lysates containing the GST fusion proteins were incubated with phosphorylated/non-phosphorylated Nuk protein. Western blots were prepared using anti-GST antibodies. The results are shown in Figures 15 and 16, and they are summarized below in Table 2.

20

Table 2

Fusion Protein	Nuk	
	<u>Non-phosphorylated</u>	<u>Phosphorylated</u>
GST	-	-
25 p85N	+/-	+/-

- 65 -

	p85C	-	+
	PLC γ N	-	++
	PLC γ C	++	++++
	GAPN	+	+++
5	Src	+	++++
	Crk	-	+
	Abl	-	+
	SH2	-	-
	Grb2	No	No

10 As shown in Table 2, phosphorylated Nuk protein bound to fusion proteins containing SH2 domains of PLC γ N, PLC γ C, Src, and GAPN.

EXAMPLE 13

GST-fusion mixing experiments with Nuk *in vitro* phosphorylation

15 The experiments described in Example 12 were also carried out in the presence of a competing phosphorylated peptide-GMKT_pYIDPFT_pYEDPNEAVR(K) from the Nuk membrane proximal region. In particular, Nuk from COS cells obtained with Nuk antisera was phosphorylated by incubation with ATP. The phosphorylated Nuk was incubated with bacterial lysates from SH2-GST fusion protein expressing
 20 bacteria, in the presence or absence of the competing peptide. Western blots for were prepared using anti-GST antibodies. As shown in Figure 17, the Nuk phosphorylated peptide competes *in vitro* with the interaction of GAP, PLC γ , and Src SH2 fusion proteins. Unphosphorylated peptide did not interfere with

- 66 -

the interaction nor did a nonspecific Trk SHC-binding peptide (HIAENPQY_pFSD).

EXAMPLE 14

5 The phosphorylation of proteins after Elk-ligand stimulation in Nuk expressing COS cells was investigated. Anti-phosphotyrosine Western blots were prepared from whole cell lysates of COS cells stimulated with Elk-ligand. The results are shown in Figure 18. Proteins of about 160, 130 (probably Nuk), 58 and 40 KD were found to be phosphorylated on tyrosine after Elk-ligand stimulation.

10 Having illustrated and described the principles of the invention in a preferred embodiment, it should be appreciated to those skilled in the art that the invention can be modified in arrangement and detail without departure from such principles. We claim all modifications coming within the scope of the following claims.

15 All publications, patents and patent applications referred to herein are incorporated by reference in their entirety to the same extent as if each individual publication, patent or patent application was specifically and individually indicated to be incorporated by reference in its entirety.

The following sequence listings form part of the application.

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SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANTS:

(A) NAME: Mount Sinai Hospital
(B) STREET: 600 University Avenue
(C) CITY: Toronto
(D) STATE: Ontario
(E) COUNTRY: Canada
(F) POSTAL CODE (ZIP):
(G) TELEPHONE NO.: (416) 586-8225
(H) TELEFAX NO.: (416) 586-8844

(A) NAME: Anthony Pawson
(B) STREET: 34 Glenwood Avenue
(C) CITY: Toronto
(D) STATE: Ontario
(E) COUNTRY: Canada
(F) POSTAL CODE (ZIP): M6P 3C6

(A) NAME: Mark Henkemeyer
(B) STREET: 263 Adelaide Street West, Apt. 503
(C) CITY: Toronto
(D) STATE: Ontario
(E) COUNTRY: Canada
(F) POSTAL CODE (ZIP): M5T 1K5

(A) NAME: Kenneth Letwin
(B) STREET: 20 Carluke Crescent, Apt. 1010
(C) CITY: Willowdale
(D) STATE: Ontario
(E) COUNTRY: Canada
(F) POSTAL CODE (ZIP): M2L 2J1

(ii) TITLE OF INVENTION: NOVEL NEURAL KINASE AND RECEPTOR
TYROSINE KINASE

(iii) NUMBER OF SEQUENCES: 2

(iv) CORRESPONDENCE ADDRESS:

(A) ADDRESSEE: Bereskin & Parr
(B) STREET: 40 King Street West, Box 401
(C) CITY: Toronto
(D) STATE: Ontario
(E) COUNTRY: Canada
(F) ZIP: M5H 3Y2

(v) COMPUTER READABLE FORM:

(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.25

(vi) CURRENT APPLICATION DATA:

(A) APPLICATION NUMBER: PCT

- 68 -

- (B) FILING DATE:
(C) CLASSIFICATION:

(viii) ATTORNEY/AGENT INFORMATION:

- (A) NAME: Kurdydyk, Linda M
(B) REGISTRATION NUMBER: 34,971
(C) REFERENCE/DOCKET NUMBER: 3153-148

(ix) TELECOMMUNICATION INFORMATION:

- (A) TELEPHONE: (416) 364-7311
(B) TELEFAX: (416) 361-1398
(C) TELEX: 06-23115

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 3105 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(vi) ORIGINAL SOURCE:

- (A) ORGANISM: Mus musculus
(D) DEVELOPMENTAL STAGE: Embryo

(vii) IMMEDIATE SOURCE:

- (A) LIBRARY: lambda gt10 cDNA library
(B) CLONE: Combined PnUKRACE A2 and K2 AND cDNA clones

(viii) POSITION IN GENOME:

- (A) CHROMOSOME/SEGMENT: Distal end of chromosome 4
(B) MAP POSITION: near the ahd-1 mutation

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

ATGGGAGCCC GGGTCCCCGT TCTGCCCGGG CTGGATGGCT CATTCTGCTG GCTGCTGCTG	60
CTGCCGCTGC TAGCCGCCGT GGAAGAAACC CTGATGGACT CTACGACAGC AACGGCTGAG	120
CTGGGCTGGA TGGTACATCC CCCATCAGGG TGGGAAGAGG TGAGCGGCTA CGACGAGAAC	180
ATGAACACTA TCCGTACCTA CCAGGTGTGC AATGTCTTTG AGTCAAGCCA GAACAAC TGG	240
CTGCCGACCA AATTCATCCG GCGCCGTGGC GCCCACC GTA TCCACGTGGA GATGAAGTTC	300
TCGGTGCGTG ACTGCAGCAG CATTCCCAGC GTGCCGGGCT CCTGCAAGGA GACCTTCAAC	360
CTCTACTACT ATGAGGCTGA TTTTGACTTA GCCACCAAAA CCTTTCCCAA CTGGATGGAG	420
AATCCGTGGG TGAAGGTGGA CACCATCGCG GCCGATGAGA GCTTCTCTCA GGTGGACCTG	480
GGTGGCCGCG TCATGAAAAT CAACACTGAG GTGCGAAGCT TCGGTCCTGT GTCCCGCAAT	540
GGTTTCTACC TGGCCTTCCA GGA CTACGGC GGCTGTATGT CCCTCATTGC TGTGCGCGTC	600

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TTCTACCGGA AGTGCCCCCG AATCATCCAG AATGGTGCCA TCTTCCAGGA GACACTATCG	660
GGGGCTGAGA GCACTTCGCT GGTGGCAGCT CGGGGCAGCT GCATCGCCAA TGCTGAAGAA	720
GTGGACGTGC CCATCAAACCT CTACTGTAAC GGGGACGGCG AATGGCTGGT GCCCATCGGT	780
CGCTGCATGT GCAAGGCGGG CTTCGAGGCT GTGGAGAACG GCACCGTCTG CCGAGGTTGT	840
CCATCAGGAA CCTTCAAGGC CAACCAAGGG GACGAAGCCT GCACCCACTG TCCCATCAAC	900
AGCCGCACCA CCTCTGAGGG TGCCACCAAC TGTGTATGCC GCAACGGCTA CTACAGGGCC	960
GACCTGGACC CCTTAGACAT GCCTTGACAC ACCATCCCCT CTGCGCCCCA GGCTGTGATC	1020
TCCAGCGTCA ACGAGACATC CCTCATGCTA GAGTGGACCC CACCCCGAGA CTCCGGGGGT	1080
CGCGAGGATC TTGTTTACAA CATCATCTGC AAGAGCTGTG GCTCCGGCCG GGGCGCATGC	1140
ACGCGCTGCG GGGACAACGT GCAGTACGCG CCCC GCCAGC TGGGCCTGAC TGAGCCGCGC	1200
ATCTACATCA GTGACCTGCT GGCACACACG CAGTACACCT TCGAGATCCA GGCCGTGAAT	1260
GGTGTGACCG ACCAGAGTCC CTTCTCACCT CAGTTCGCCT CTGTGAACAT CACCACCAAC	1320
CAAGCAGCAC CATCGGCCGT GTCCATCATG CACCAGGTGA GCCGCACTGT GGACAGCATC	1380
ACCCTGTGCT GGTCCCAGCC AGACCAGCCC AACGGTGTGA TCCTGGACTA CGAGCTGCAG	1440
TACTATGAGA AGGAGCTCAG TGAGTACAAC GCCACGGCCA TAAAAAGCCC CACCAACACA	1500
GTCAGTGTGC AGGGCCTCAA AGCCGGCGCC ATCTATGTCT TCCAGGTGCG GGCACGCACC	1560
GTTGCAGGCT ATGGGCGCTA CAGTGGCAAG ATGTACTTCC AAACCATGAC AGAAGCCGAG	1620
TACCAGACCA GCATCAAGGA AAAGCTACCC CTCATCGTTG GCTCCTCCGC CGCCGGCTTA	1680
GTCTTCCTCA TCGCTGTGGT CGTCATTGCC ATCGTATGTA ACAGACGGGG GTTTGAGCGT	1740
GCCGACTCAG AGTACACGGA CAAGCTACAG CACTACACCA GCGGACACAT GACCCAGGC	1800
ATGAAGATCT ATATAGATCC TTTCACCTAT GAAGATCCTA ATGAGGCACT GCGGGAGTTT	1860
GCCAAGGAAA TTGACATCTC CTGTGTCAAG ATTGAGCAGG TGATTGGAGC AGGGGAATTT	1920
GGTGAGGTCT GCAGTGGCCA TTTGAAGCTG CCAGGCAAGA GAGAGATCTT TGTAGCCATC	1980
AAGACCCTCA AGTCAGGATA CACGGAGAAA CAGCGCCGGG ACTTCCTGAG TGAGGCATCC	2040
ATCATGGGCC AGTTCGACCA CCCCAATGTC ATCCATCTGG AAGGGGTTGT CACCAAGAGC	2100
ACACCTGTCA TGATCATCAC TGAATTCATG GAGAATGGAT CTCTGGACTC CTTCTCCGG	2160
CAAAATGATG GGCAGTTCAC AGTCATCCAA CTGGTGGGCA TGCTGAGGGG CATTGCAGCC	2220
GGCATGAAGT ACCTGGCGGA CATGAACTAC GTGCACCGTG ACCTTGCTGC TCGAAACATC	2280

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CTCGTCAACA GTAACCTGGT GTGTAAGGTG TCTGACTTTG GGCTCTCACG CTTCTGGAG 2340
 GATGACACGT CTGACCCAC CTATACCAGC GCTCTGGGTG GGAAGATCCC CATCCGTTGG 2400
 ACGGCACCGG AAGCCATCCA GTACCGGAAA TTCACCTCGG CCAGTGATGT GTGGAGCTAT 2460
 GGCATCGTCA TGTGGGAGGT GATGTCCTAC GGGGAACGAC CCTACTGGGA CATGACCAAT 2520
 CAAGACGTAA TCAACGCCAT TGAACAGGAC TACAGACTAC CTCCGCCCAT GGACTGCCCT 2580
 AGCGCCCTGC ACCAGCTCAT GCTGGACTGC TGGCAGAAGG ACCGCAACCA CCGGCCCAAG 2640
 TTCGGCCAGA TTGTCAACAC GCTGGACAAG ATGATCCGAA ACCCAACAG CCTCAAAGCC 2700
 ATGGCACCCC TGTCTCTGG CATCAACCTG CCACTGCTGG ACCGCACGAT ACCGGACTAC 2760
 ACCAGCTTTA ACACAGTGA TGACTGGCTA GAGGCCATCA AGATGGGCCA GTACAAGGAG 2820
 AGCTTTGCCA ACGCCGGCTT CACCTCTTTC GACGTTGTAT CTCAGATGAT GATGGAGGAC 2880
 ATTCTCCGCG TTGGGGTCAC TCTAGCTGGC CACCAGAAAA AAATCCTGAA CAGTATCCAG 2940
 GTGATGCGGG CCCAGATGAA CCAGATCCAG TCTGTAGAGG TTTGACATTC GCCTGCCTCG 3000
 GTTCTCCTCT TCCTCCACGC CGCCCCTGAG CCCCTACGTC GGTCCCTGCT GCTCTGTCAC 3060
 TGCAGGTCAG CACTGCCAGG AGGCCACAGA CAACAGGAAG ACCAA 3105

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 994 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(vi) ORIGINAL SOURCE:

- (A) ORGANISM: *Mus musculus*
- (D) DEVELOPMENTAL STAGE: Embryo

(vii) IMMEDIATE SOURCE:

- (A) LIBRARY: lambda gt10 cDNA library
- (B) CLONE: Combined pNukRACE A2 and K2 and cDNA clones

(viii) POSITION IN GENOME:

- (A) CHROMOSOME/SEGMENT: Distal end of chromosome 4
- (B) MAP POSITION: near the *ahd-1* mutation

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

Met Gly Ala Arg Val Pro Val Leu Pro Gly Leu Asp Gly Ser Phe Cys
 1 5 10 15

Trp Leu Leu Leu Leu Pro Leu Leu Ala Ala Val Glu Glu Thr Leu Met

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	20							25						30					
Asp	Ser	Thr	Thr	Ala	Thr	Ala	Glu	Leu	Gly	Trp	Met	Val	His	Pro	Pro				
		35					40					45							
Ser	Gly	Trp	Glu	Glu	Val	Ser	Gly	Tyr	Asp	Glu	Asn	Met	Asn	Thr	Ile				
	50					55					60								
Arg	Thr	Tyr	Gln	Val	Cys	Asn	Val	Phe	Glu	Ser	Ser	Gln	Asn	Asn	Trp				
65					70					75					80				
Leu	Arg	Thr	Lys	Phe	Ile	Arg	Arg	Arg	Gly	Ala	His	Arg	Ile	His	Val				
				85					90					95					
Glu	Met	Lys	Phe	Ser	Val	Arg	Asp	Cys	Ser	Ser	Ile	Pro	Ser	Val	Pro				
			100					105					110						
Gly	Ser	Cys	Lys	Glu	Thr	Phe	Asn	Leu	Tyr	Tyr	Tyr	Glu	Ala	Asp	Phe				
		115					120					125							
Asp	Leu	Ala	Thr	Lys	Thr	Phe	Pro	Asn	Trp	Met	Glu	Asn	Pro	Trp	Val				
	130					135					140								
Lys	Val	Asp	Thr	Ile	Ala	Ala	Asp	Glu	Ser	Phe	Ser	Gln	Val	Asp	Leu				
145					150					155					160				
Gly	Gly	Arg	Val	Met	Lys	Ile	Asn	Thr	Glu	Val	Arg	Ser	Phe	Gly	Pro				
				165					170					175					
Val	Ser	Arg	Asn	Gly	Phe	Tyr	Leu	Ala	Phe	Gln	Asp	Tyr	Gly	Gly	Cys				
			180					185					190						
Met	Ser	Leu	Ile	Ala	Val	Arg	Val	Phe	Tyr	Arg	Lys	Cys	Pro	Arg	Ile				
		195					200					205							
Ile	Gln	Asn	Gly	Ala	Ile	Phe	Gln	Glu	Thr	Leu	Ser	Gly	Ala	Glu	Ser				
	210					215					220								
Thr	Ser	Leu	Val	Ala	Ala	Arg	Gly	Ser	Cys	Ile	Ala	Asn	Ala	Glu	Glu				
225					230					235					240				
Val	Asp	Val	Pro	Ile	Lys	Leu	Tyr	Cys	Asn	Gly	Asp	Gly	Glu	Trp	Leu				
				245					250					255					
Val	Pro	Ile	Gly	Arg	Cys	Met	Cys	Lys	Ala	Gly	Phe	Glu	Ala	Val	Glu				
			260					265					270						
Asn	Gly	Thr	Val	Cys	Arg	Gly	Cys	Pro	Ser	Gly	Thr	Phe	Lys	Ala	Asn				
		275					280					285							
Gln	Gly	Asp	Glu	Ala	Cys	Thr	His	Cys	Pro	Ile	Asn	Ser	Arg	Thr	Thr				
	290					295					300								
Ser	Glu	Gly	Ala	Thr	Asn	Cys	Val	Cys	Arg	Asn	Gly	Tyr	Tyr	Arg	Ala				
305					310					315					320				

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Asp Leu Asp Pro Leu Asp Met Pro Cys Thr Thr Ile Pro Ser Ala Pro
 325 330 335
 Gln Ala Val Ile Ser Ser Val Asn Glu Thr Ser Leu Met Leu Glu Trp
 340 345 350
 Thr Pro Pro Arg Asp Ser Gly Gly Arg Glu Asp Leu Val Tyr Asn Ile
 355 360 365
 Ile Cys Lys Ser Cys Gly Ser Gly Arg Gly Ala Cys Thr Arg Cys Gly
 370 375 380
 Asp Asn Val Gln Tyr Ala Pro Arg Gln Leu Gly Leu Thr Glu Pro Arg
 385 390 395 400
 Ile Tyr Ile Ser Asp Leu Leu Ala His Thr Gln Tyr Thr Phe Glu Ile
 405 410 415
 Gln Ala Val Asn Gly Val Thr Asp Gln Ser Pro Phe Ser Pro Gln Phe
 420 425 430
 Ala Ser Val Asn Ile Thr Thr Asn Gln Ala Ala Pro Ser Ala Val Ser
 435 440 445
 Ile Met His Gln Val Ser Arg Thr Val Asp Ser Ile Thr Leu Ser Trp
 450 455 460
 Ser Gln Pro Asp Gln Pro Asn Gly Val Ile Leu Asp Tyr Glu Leu Gln
 465 470 475 480
 Tyr Tyr Glu Lys Glu Leu Ser Glu Tyr Asn Ala Thr Ala Ile Lys Ser
 485 490 495
 Pro Thr Asn Thr Val Thr Val Gln Gly Leu Lys Ala Gly Ala Ile Tyr
 500 505 510
 Val Phe Gln Val Arg Ala Arg Thr Val Ala Gly Tyr Gly Arg Tyr Ser
 515 520 525
 Gly Lys Met Tyr Phe Gln Thr Met Thr Glu Ala Glu Tyr Gln Thr Ser
 530 535 540
 Ile Lys Glu Lys Leu Pro Leu Ile Val Gly Ser Ser Ala Ala Gly Leu
 545 550 555 560
 Val Phe Leu Ile Ala Val Val Val Ile Ala Ile Val Cys Asn Arg Arg
 565 570 575
 Gly Phe Glu Arg Ala Asp Ser Glu Tyr Thr Asp Lys Leu Gln His Tyr
 580 585 590
 Thr Ser Gly His Met Thr Pro Gly Met Lys Ile Tyr Ile Asp Pro Phe
 595 600 605
 Thr Tyr Glu Asp Pro Asn Glu Ala Val Arg Glu Phe Ala Lys Glu Ile
 610 615 620

- 73 -

Asp Ile Ser Cys Val Lys Ile Glu Gln Val Ile Gly Ala Gly Glu Phe
 625 630 635 640
 Gly Glu Val Cys Ser Gly His Leu Lys Leu Pro Gly Lys Arg Glu Ile
 645 650 655
 Phe Val Ala Ile Lys Thr Leu Lys Ser Gly Tyr Thr Glu Lys Gln Arg
 660 665 670
 Arg Asp Phe Leu Ser Glu Ala Ser Ile Met Gly Gln Phe Asp His Pro
 675 680 685
 Asn Val Ile His Leu Glu Gly Val Val Thr Lys Ser Thr Pro Val Met
 690 695 700
 Ile Ile Thr Glu Phe Met Glu Asn Gly Ser Leu Asp Ser Phe Leu Arg
 705 710 715 720
 Gln Asn Asp Gly Gln Phe Thr Val Ile Gln Leu Val Gly Met Leu Arg
 725 730 735
 Gly Ile Ala Ala Gly Met Lys Tyr Leu Ala Asp Met Asn Tyr Val His
 740 745 750
 Arg Asp Leu Ala Ala Arg Asn Ile Leu Val Asn Ser Asn Leu Val Cys
 755 760 765
 Lys Val Ser Asp Phe Gly Leu Ser Arg Phe Leu Glu Asp Asp Thr Ser
 770 775 780
 Asp Pro Thr Tyr Thr Ser Ala Leu Gly Gly Lys Ile Pro Ile Arg Trp
 785 790 795 800
 Thr Ala Pro Glu Ala Ile Gln Tyr Arg Lys Phe Thr Ser Ala Ser Asp
 805 810 815
 Val Trp Ser Tyr Gly Ile Val Met Trp Glu Val Met Ser Tyr Gly Glu
 820 825 830
 Arg Pro Tyr Trp Asp Met Thr Asn Gln Asp Val Ile Asn Ala Ile Glu
 835 840 845
 Gln Asp Tyr Arg Leu Pro Pro Pro Met Asp Cys Pro Ser Ala Leu His
 850 855 860
 Gln Leu Met Leu Asp Cys Trp Gln Lys Asp Arg Asn His Arg Pro Lys
 865 870 875 880
 Phe Gly Gln Ile Val Asn Thr Leu Asp Lys Met Ile Arg Asn Pro Asn
 885 890 895
 Ser Leu Lys Ala Met Ala Pro Leu Ser Ser Gly Ile Asn Leu Pro Leu
 900 905 910
 Leu Asp Arg Thr Ile Pro Asp Tyr Thr Ser Phe Asn Thr Val Asp Glu
 915 920 925

- 74 -

Trp Leu Glu Ala Ile Lys Met Gly Gln Tyr Lys Glu Ser Phe Ala Asn
930 935 940

Ala Gly Phe Thr Ser Phe Asp Val Val Ser Gln Met Met Met Glu Asp
945 950 955 960

Ile Leu Arg Val Gly Val Thr Leu Ala Gly His Gln Lys Lys Ile Leu
965 970 975

Asn Ser Ile Gln Val Met Arg Ala Gln Met Asn Gln Ile Gln Ser Val
980 985 990

Glu Val

WE CLAIM:

1. An isolated and purified nucleic acid molecule comprising:
 - (a) a nucleic acid sequence encoding a protein having the amino acid sequence as shown in SEQ ID NO:2 and Figure 2;
 - 5 (b) nucleic acid sequences complementary to (a);
 - (c) nucleic acid sequences which are at least 97% homologous to (a);or,
 - (d) a fragment of (a) or (b) that is at least 15 bases and which will hybridize to (a) or (b) under stringent hybridization conditions.
- 10 2. A purified and isolated nucleic acid molecule as claimed in claim 1 comprising:
 - (a) a nucleic acid sequence as shown in SEQ ID NO:1 and Figure 1, wherein T can also be U;
 - (b) nucleic acid sequences complementary to (a);
 - 15 (c) nucleic acid sequences which are at least 85% homologous to (a);or,
 - (d) a fragment of (a) or (b) that is at least 15 bases and which will hybridize to (a) or (b) under stringent hybridization conditions.
- 20 3. A purified and isolated receptor tyrosine kinase protein having an amino acid sequence as shown in SEQ ID NO:2 or a sequence having at least 97% homology thereto, or an isoform or a part of the protein having at least 20 amino acids.
- 25 4. A part of the protein as claimed in claim 3 comprising an extracellular domain of a tyrosine kinase having the amino acid sequence as shown in SEQ ID NO: 2 from amino acid number 26 to 548 or a sequence having at least 97% homology thereto.
5. A part of the protein as claimed in claim 3 comprising a carboxy

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terminal of a tyrosine kinase protein having the amino acid sequence as shown in SEQ ID NO: 2 from amino acid number 601 to 994 or a sequence having at least 97% homology thereto.

6. A recombinant molecule comprising a nucleic acid molecule as claimed in claim 1 and one or more transcription or translation elements operatively linked thereto.
7. A transformant host cell including a recombinant molecule comprising a nucleic acid molecule as claimed in claim 1 and one or more transcription or translation elements operatively linked thereto.
8. A method for preparing a receptor tyrosine kinase protein comprising expressing a purified and isolated nucleic acid molecule as claimed in claim 1 in a host cell.
9. A method for identifying a substance which is capable of binding to a receptor tyrosine kinase protein or an isoform or part thereof as claimed in claim 3 comprising reacting the receptor tyrosine kinase protein or an isoform or part thereof, under conditions which permit the formation of a complex between the substance and the receptor tyrosine kinase protein, or isoform or part thereof, assaying for substance-receptor complexes, for free substance, for non-complexed receptor tyrosine kinase protein, or isoforms or parts thereof, or for activation of the receptor tyrosine kinase protein.
10. The method as claimed in claim 9, wherein the substance is a ligand of the receptor tyrosine kinase protein which is capable of activating the receptor tyrosine kinase.
11. The method as claimed in claim 9 wherein the part of the receptor tyrosine kinase protein has the amino acid sequence as shown in SEQ ID NO: 2 from amino acid number 26 to 548 or a sequence having at least 97 %

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homology thereto.

12. A method for assaying a medium for the presence of an agonist or antagonist of the interaction of a receptor tyrosine kinase protein as claimed in claim 3 and a substance which is capable of binding to the receptor tyrosine
5 kinase protein, which comprises providing a known concentration of the receptor tyrosine kinase protein, reacting the receptor tyrosine kinase protein with a substance which is capable of binding to the receptor tyrosine kinase protein and a suspected agonist or antagonist under conditions which permit the formation of substance-receptor tyrosine kinase protein complexes, and
10 assaying for substance-receptor tyrosine kinase protein complexes, for free substance, for non-complexed proteins, or for activation of the receptor tyrosine kinase protein.

13. A method for identifying a substance which is capable of binding to an activated receptor tyrosine kinase protein as claimed in claim 3, or isoform
15 or part thereof which is activated comprising reacting a receptor tyrosine kinase protein as claimed in claim 3 which is activated, or an isoform or part of the protein, with at least one substance which potentially can bind with the receptor tyrosine kinase protein, isoform or part of the protein, under conditions which permit the formation of substance-receptor kinase protein
20 complexes, and assaying for substance-receptor kinase protein complexes, for free substance, for non-complexed receptor kinase proteins, or for phosphorylation of the substance.

14. A monoclonal or polyclonal antibody specific for an epitope of a protein as claimed in claim 3.

25 15. A recombinant DNA molecule adapted for transformation of a host cell comprising a nucleic acid molecule as claimed in claim 2 wherein a portion of the nucleic acid sequence is deleted, and one or more transcription or translation elements operatively linked thereto.

16. A transgenic non-human mammal all of whose germ cells and somatic cells contain a recombinant DNA molecule as claimed in claim 15 introduced into the animal, or an ancestor of the mammal at an embryonic stage.
- 5

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FIGURE 1

ATGGGAGCCCCGGGTCCCCGTTCTGCCCCGGGCTGGATGGCTCATTCTGCTGGCTGCTGCTG	60
CTGCCGCTGCTAGCCGCCGTGGAAGAAACCCTGATGGACTCTACGACAGCAACGGCTGAG	120
CTGGGCTGGATGGTACATCCCCCATCAGGGTGGGAAGAGGTGAGCGGCTACGACGAGAAC	180
ATGAACACTATCCGTACCTACCAGGTGTGCAATGTCTTTGAGTCAAGCCAGAACAACCTGG	240
CTGCGGACCAAATTCATCCGGCGCCGTGGCGCCCACCGTATCCACGTGGAGATGAAGTTC	300
TCGGTGCGTGACTGCAGCAGCATTCCCAGCGTGCCGGGCTCCTGCAAGGAGACCTTCAAC	360
CTCTACTACTATGAGGCTGATTTTGACTTAGCCACCAAAACCTTTCCCAACTGGATGGAG	420
AATCCGTGGGTGAAGGTGGACACCATCGCGGCCGATGAGAGCTTCTCTCAGGTGGACCTG	480
GGTGGCCGCGTCATGAAAATCAAACTGAGGTGCGAAGCTTCGGTCCTGTGTCCCGCAAT	540
GGTTTCTACCTGGCCTTCCAGGACTACGGCGGCTGTATGTCCCTCATTGCTGTGCGCGTC	600
TTCTACCGGAAGTGCCCCCGAATCATCCAGAATGGTGCCATCTTCCAGGAGACACTATCG	660
GGGGCTGAGAGCACTTCGCTGGTGGCAGCTCGGGGCAGCTGCATCGCCAATGCTGAAGAA	720
GTGGACGTGCCCATCAAACCTCTACTGTAACGGGGACGGCGAATGGCTGGTGCCCATCGGT	780
CGCTGCATGTGCAAGGCGGGCTTCGAGGCTGTGGAGAACGGCACCGTCTGCCGAGGTTGT	840
CCATCAGGAACCTTCAAGGCCAACCAAGGGGACGAAGCCTGCACCCACTGTCCCATCAAC	900
AGCCGCACCACTCTGAGGGTGCCACCAACTGTGTATGCCGCAACGGCTACTACAGGGCC	960
GACCTGGACCCCTTAGACATGCCTTGACACAACCATCCCCTCTGCGCCCCAGGCTGTGATC	1020
TCCAGCGTCAACGAGACATCCCTCATGCTAGAGTGGACCCACCCGAGACTCCGGGGGT	1080
CGCGAGGATCTTGTTTACAACATCATCTGCAAGAGCTGTGGCTCCGGCCGGGGCGCATGC	1140
ACGCGCTGCGGGGACAACGTGCAGTACGCGCCCCGCCAGCTGGGCCTGACTGAGCCGCGC	1200
ATCTACATCAGTGACCTGCTGGCACACACGCAGTACACCTTCGAGATCCAGGCCGTGAAT	1260
GGTGTGACCGACCAGAGTCCCTTCTCACCTCAGTTCGCCTCTGTGAACATCACCACCAAC	1320
CAAGCAGCACCATCGGCCGTGTCCATCATGCACCAGGTGAGCCGCACTGTGGACAGCATC	1380
ACCTGTGCTGGTCCCAGCCAGACCAGCCCAACGGTGTGATCCTGGACTACGAGCTGCAG	1440
TACTATGAGAAGGAGCTCAGTGAGTACAACGCCACGGCCATAAAAAGCCCCACCAACACA	1500
GTCAGTGTGCAGGGCCTCAAAGCCGGCGCCATCTATGTCTTCCAGGTGCGGGCACGCACC	1560

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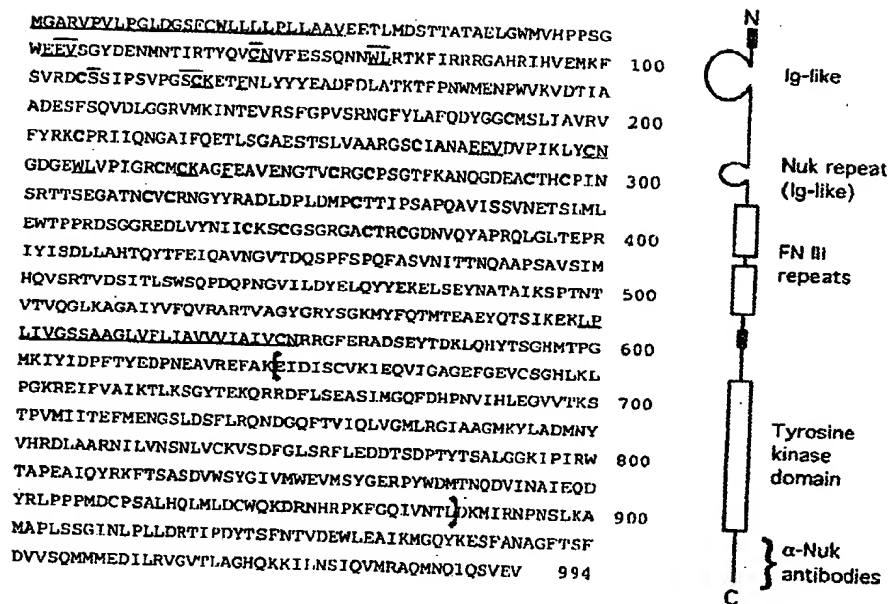
FIGURE 1 CONT'D

GTTGCAGGCTATGGGCGCTACAGTGGCAAGATGTACTTCCAAACCATGACAGAAGCCGAG	1620
TACCAGACCAGCATCAAGGAAAAGCTACCCCTCATCGTTGGCTCCTCCGCCGCCGGCTTA	1680
GTCTTCCTCATCGCTGTGGTCGTATTGCCATCGTATGTAACAGACGGGGGTTTGAGCGT	1740
GCCGACTCAGAGTACACGGACAAGCTACAGCACTACACCAGCGGACACATGACCCCAGGC	1800
ATGAAGATCTATATAGATCCTTTCACCTATGAAGATCCTAATGAGGCAGTGCGGGAGTTT	1860
GCCAAGGAAATTGACATCTCCTGTGTCAAGATTGAGCAGGTGATTGGAGCAGGGGAATTT	1920
GGTGAGGTCTGCAGTGGCCATTTGAAGCTGCCAGGCAAGAGAGAGATCTTTGTAGCCATC	1980
AAGACCCTCAAGTCAGGATACACGGAGAAACAGCGCCGGGACTTCCTGAGTGAGGCATCC	2040
ATCATGGGCCAGTTTCGACCACCCCAATGTCATCCATCTGGAAGGGGTGTACCAAGAGC	2100
ACACCTGTCATGATCATCACTGAATTCATGGAGAATGGATCTCTGGACTCCTTCCTCCGG	2160
CAAAATGATGGGCAGTTCACAGTCATCCAACTGGTGGGCATGCTGAGGGGCATTGCAGCC	2220
GGCATGAAGTACCTGGCGGACATGAACTACGTGCACCGTGACCTTGCTGCTCGAAACATC	2280
CTCGTCAACAGTAACCTGGTGTGTAAGGTGTCTGACTTTGGGCTCTCACGCTTCCTGGAG	2340
GATGACACGTCTGACCCACCTATACCAGCGCTCTGGGTGGGAAGATCCCCATCCGTTGG	2400
ACGGCACCGGAAGCCATCCAGTACCGGAAATTCACCTCGGCCAGTGATGTGTGGAGCTAT	2460
GGCATCGTCATGTGGGAGGTGATGTCCTACGGGGAACGACCCTACTGGGACATGACCAAT	2520
CAAGACGTAATCAACGCCATTGAACAGGACTACAGACTACCTCCGCCCATGGACTGCCCT	2580
AGCGCCCTGCACCAGCTCATGCTGGACTGCTGGCAGAAGGACCGCAACCACCGGCCCAAG	2640
TTCGGCCAGATTGTCAACACGCTGGACAAGATGATCCGAAACCCCAACAGCCTCAAAGCC	2700
ATGGCACCCCTGTCCTCTGGCATCAACCTGCCACTGCTGGACCGCACGATACCGGACTAC	2760
ACCAGCTTTAACACAGTGGATGAGTGGCTAGAGGCCATCAAGATGGGCCAGTACAAGGAG	2820
AGCTTTGCCAACGCCGGCTTCACCTCTTTGACGTTGTATCTCAGATGATGATGGAGGAC	2880
ATTCTCCGCGTTGGGGTCACTCTAGCTGGCCACCAGAAAAAATCCTGAACAGTATCCAG	2940
GTGATGCGGGCCAGATGAACCAGATCCAGTCTGTAGAGGTTTGACATTGCGCTGCCTCG	3000
GTTCTCCTCTTCCTCCACGCCGCCCTGAGCCCCTACGTCCGTCCCTGCTGCTCTGTAC	3060
TGCAGGTCAGCACTGCCAGGAGGCCACAGACAACAGGAAGACCAA	3105

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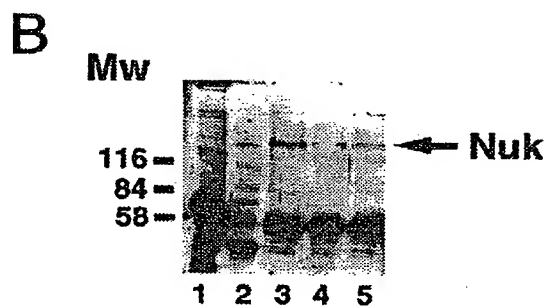
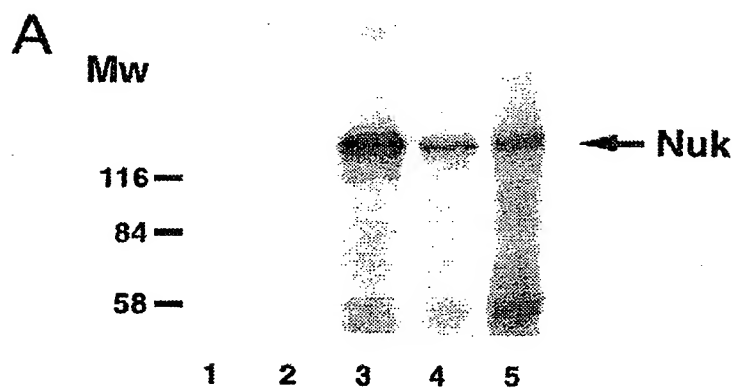
FIGURE 2



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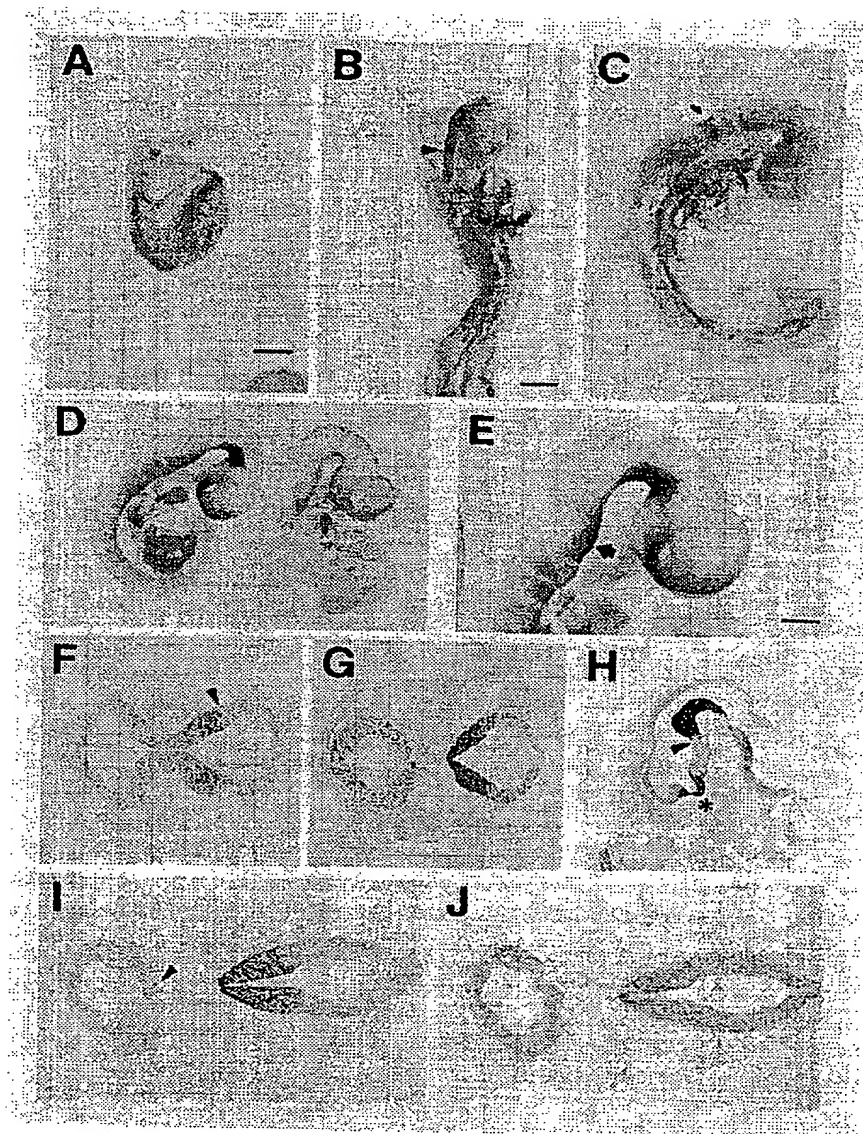
FIGURES 3 A-B



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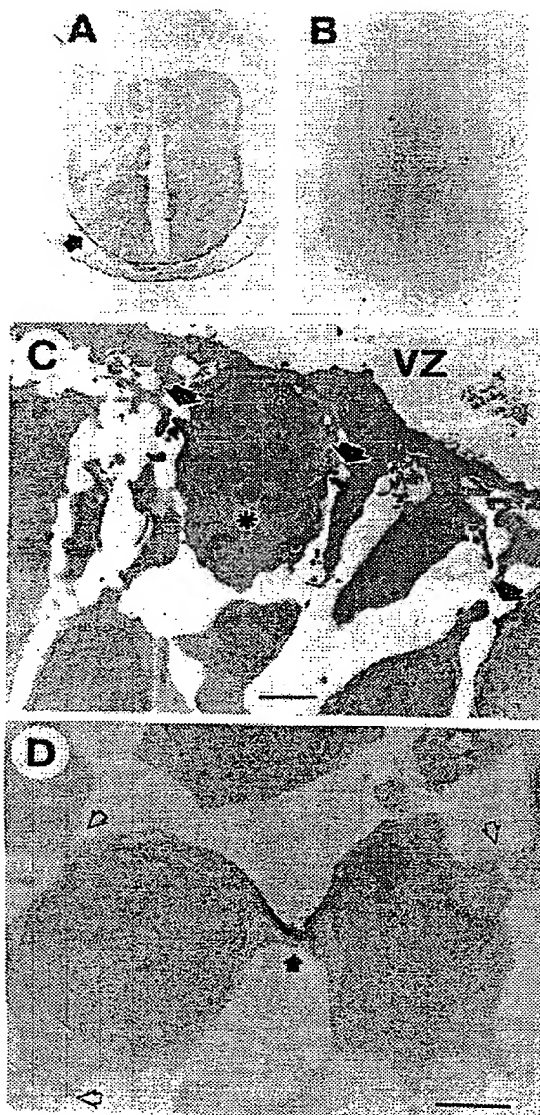
FIGURES 4 A-J



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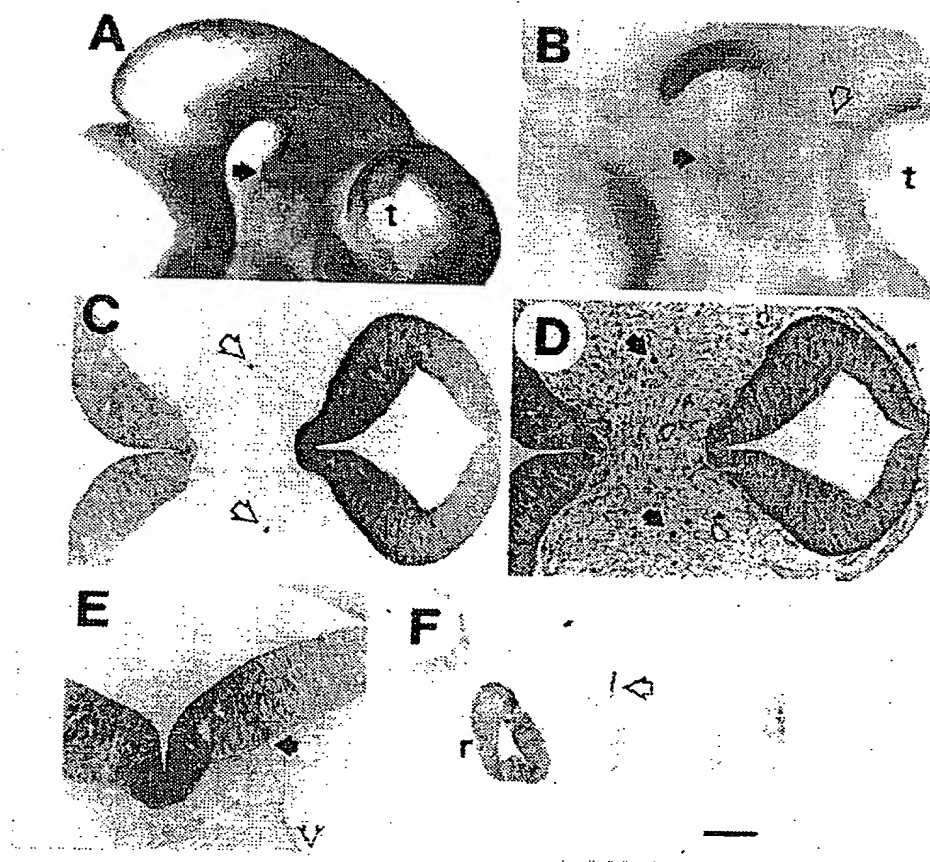
FIGURES 5 A-D



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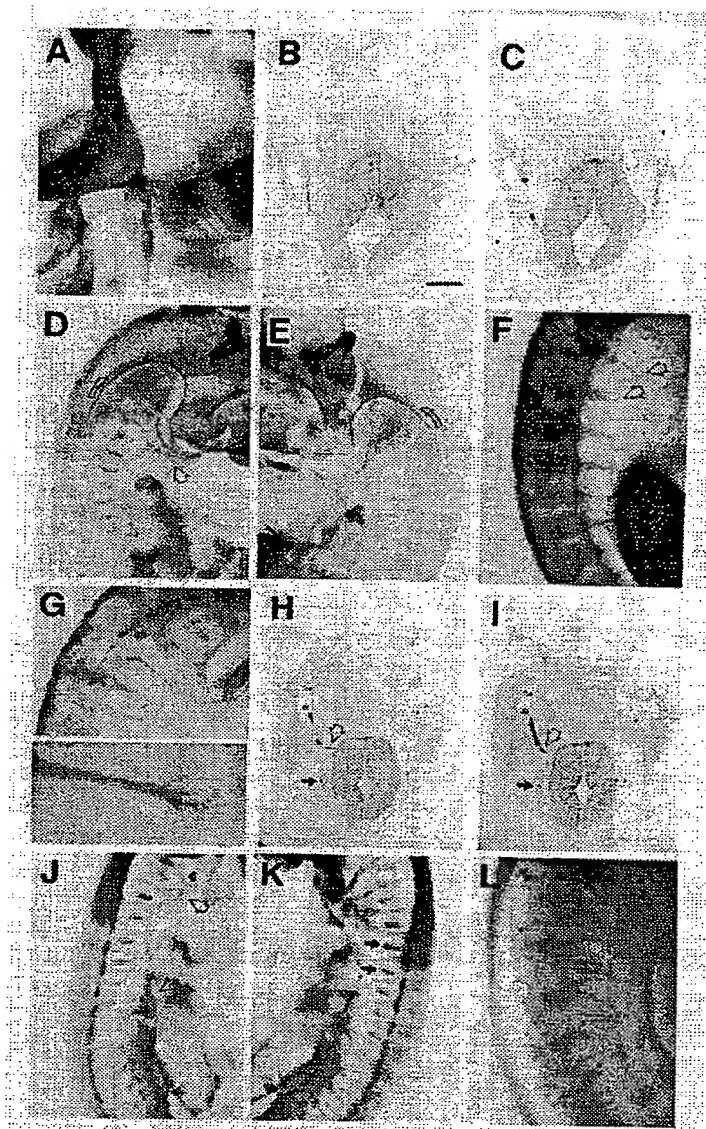
FIGURES 6 A-F



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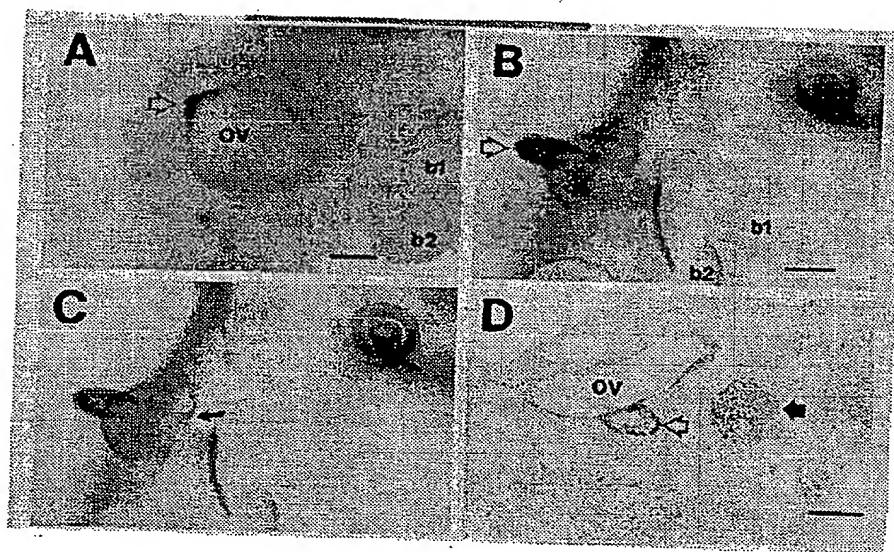
FIGURES 7 A-L



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FIGURES 8 A-D

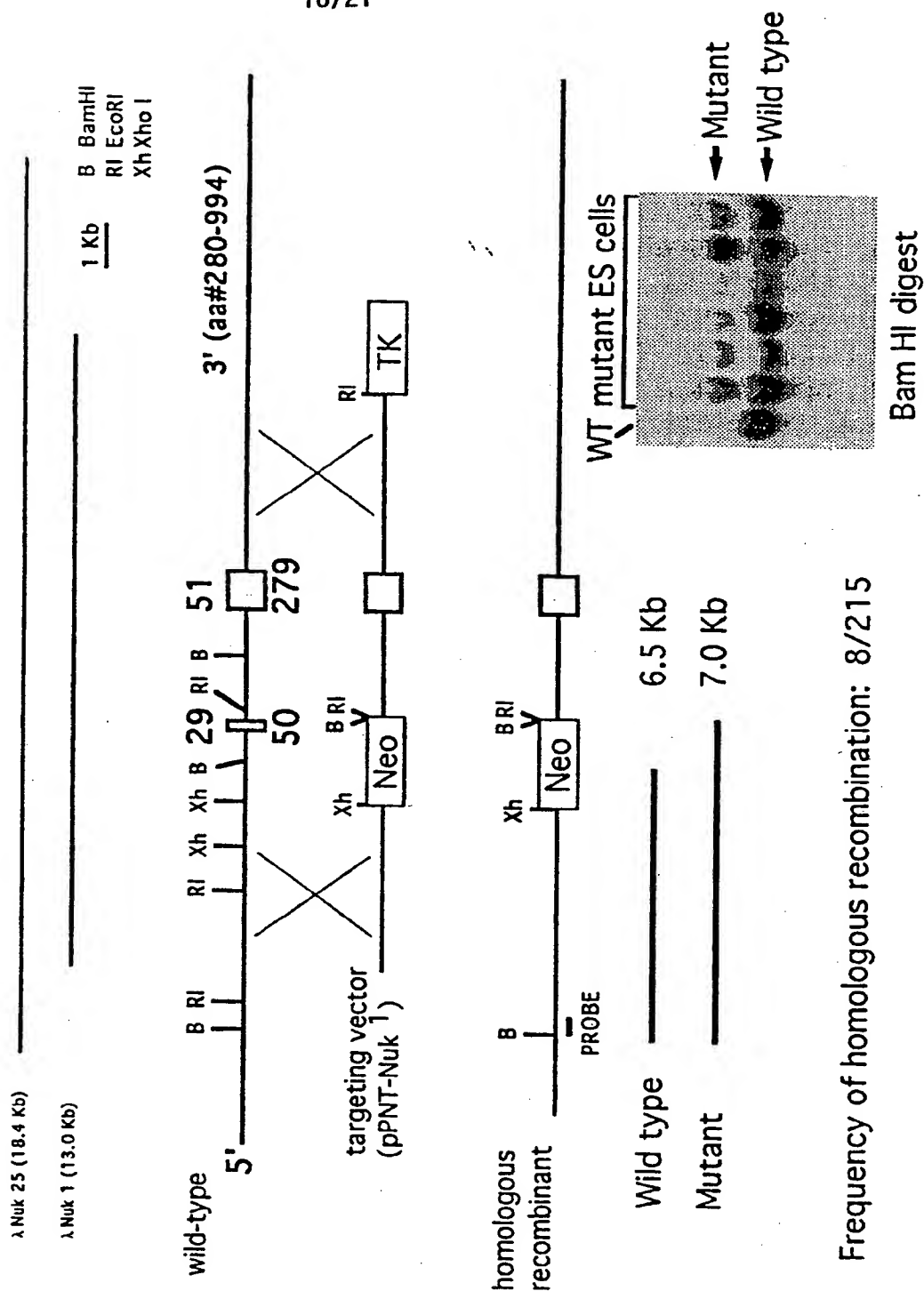


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FIGURE 9

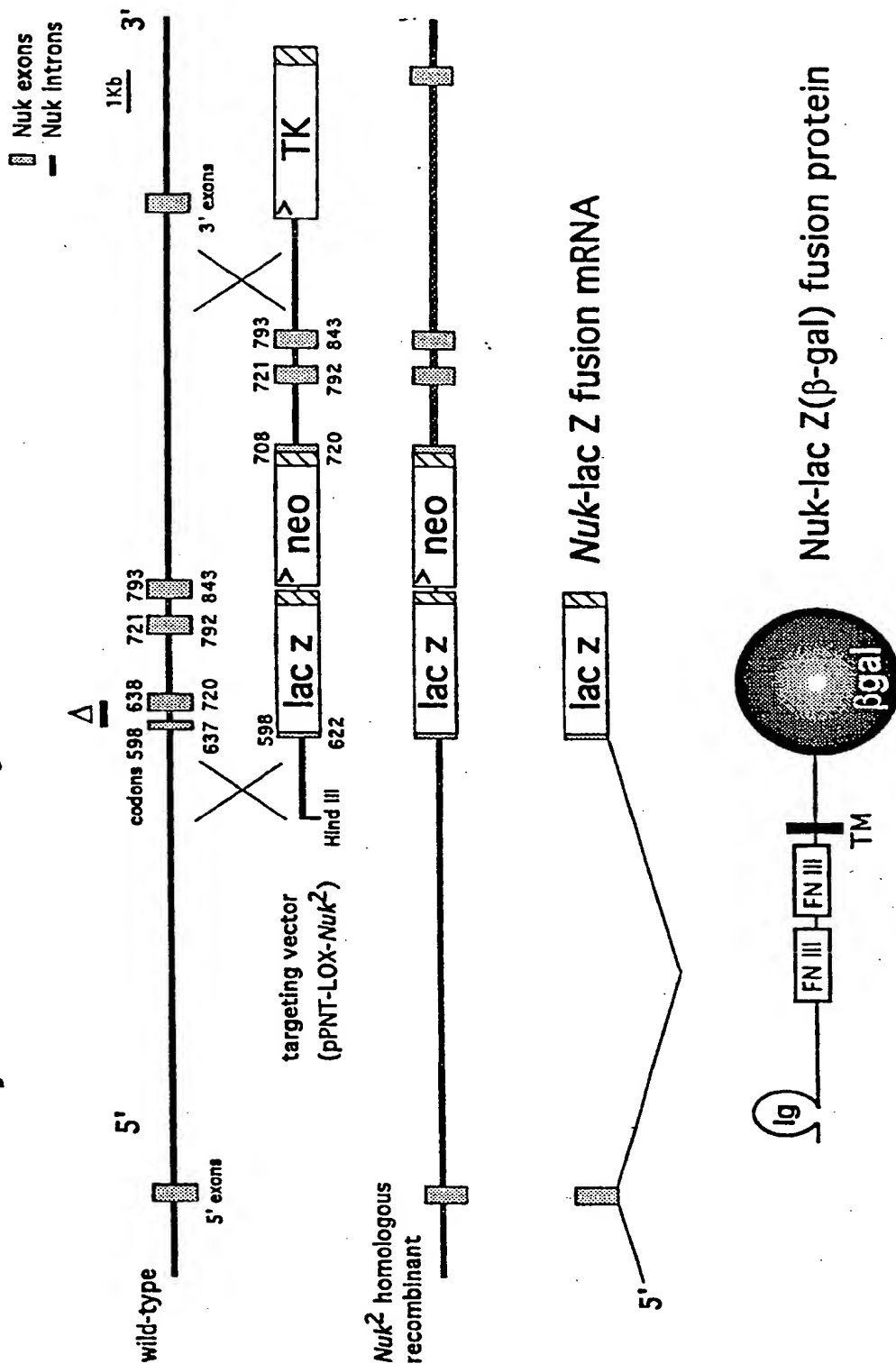
Nuk gene targeting: deletion of exon 2 (codons 29 to 50).



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FIGURE 10

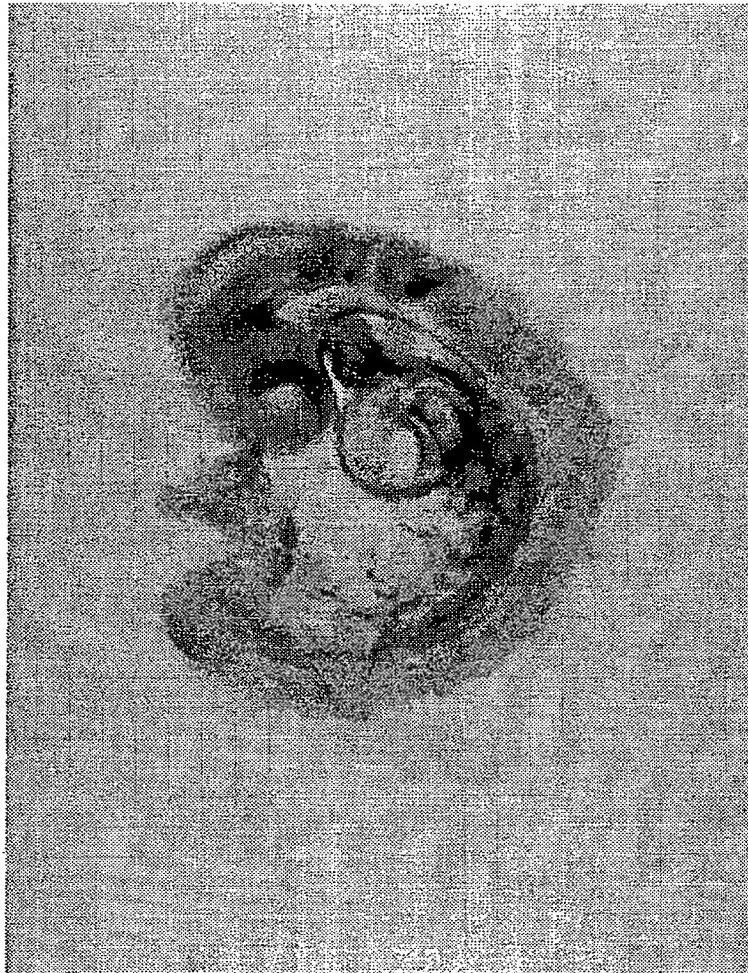
The *Nuk*² mutation expresses a Nuk-βgal fusion protein lacking the tyrosine kinase catalytic domain.



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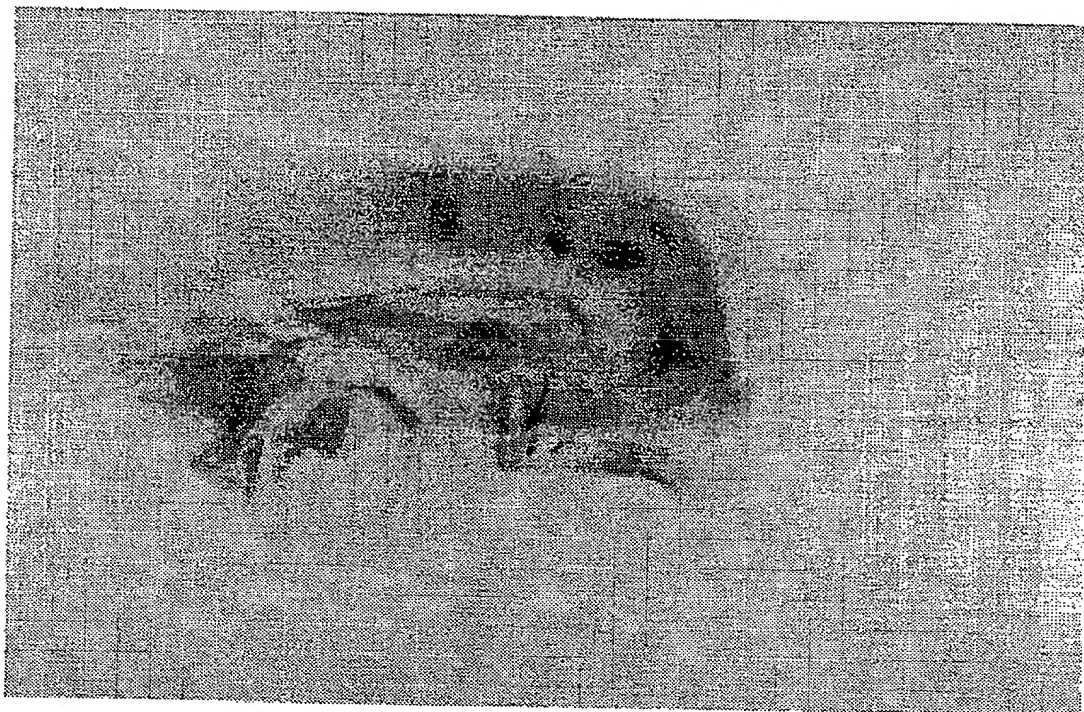
FIGURE 11 A



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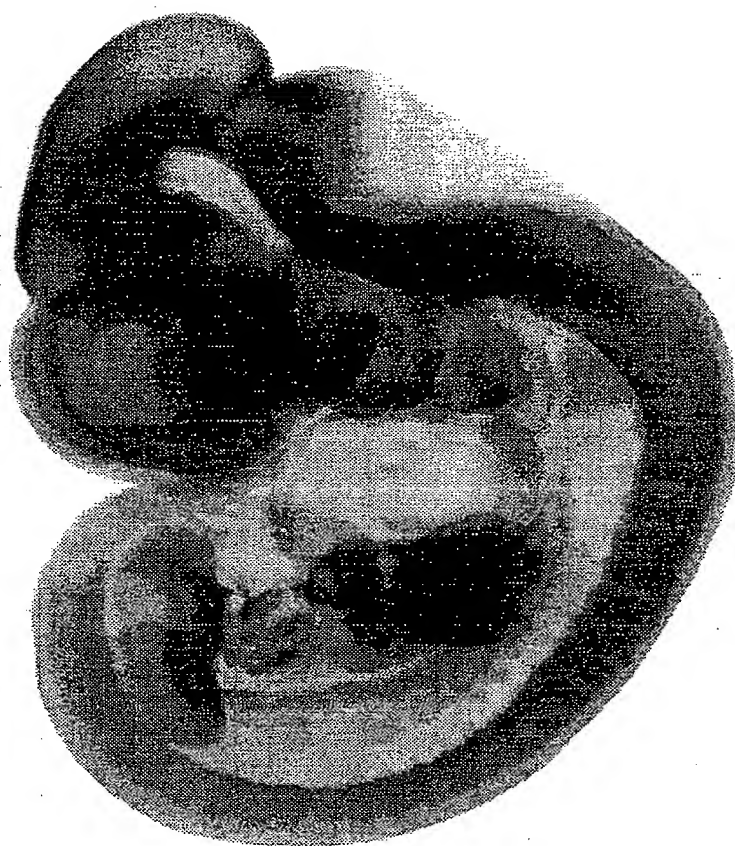
FIGURE 11 B



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FIGURE 12 A



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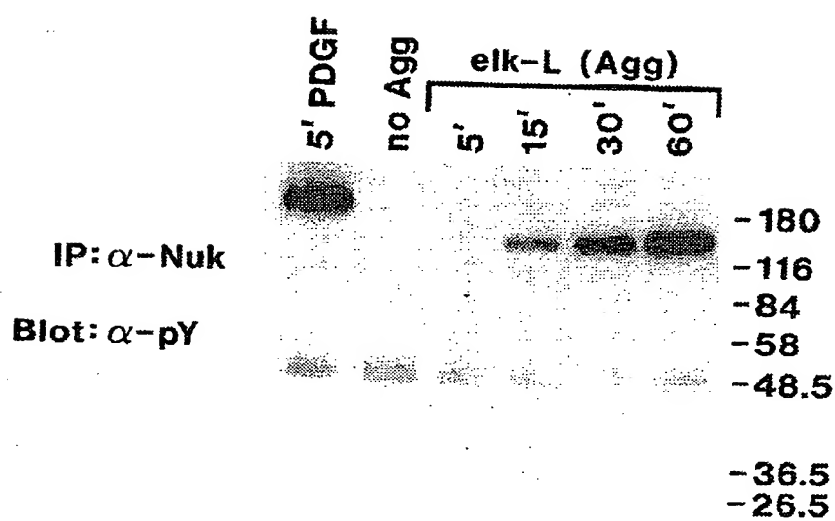
FIGURE 12 B



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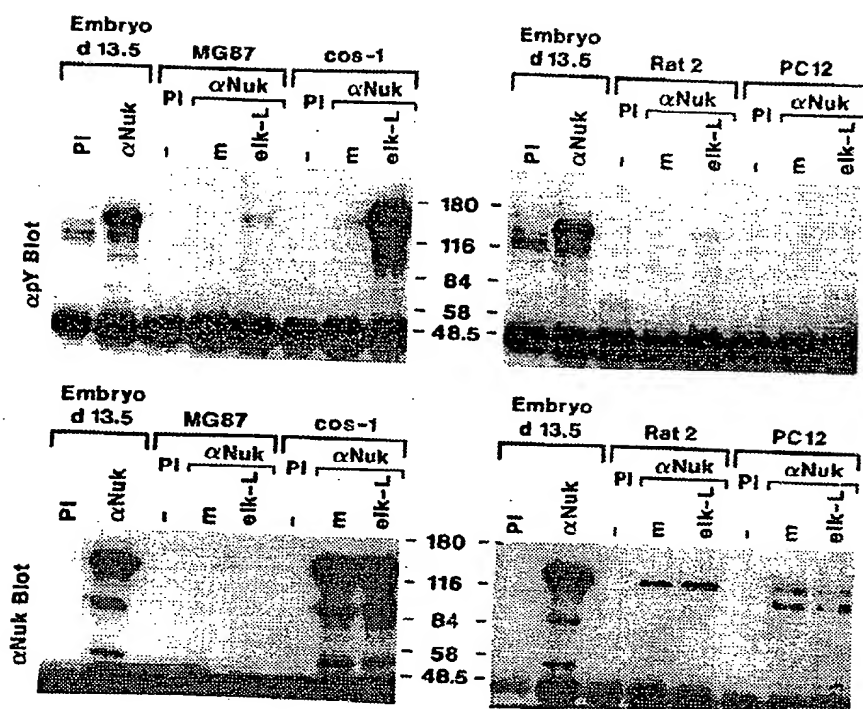
FIGURE 13



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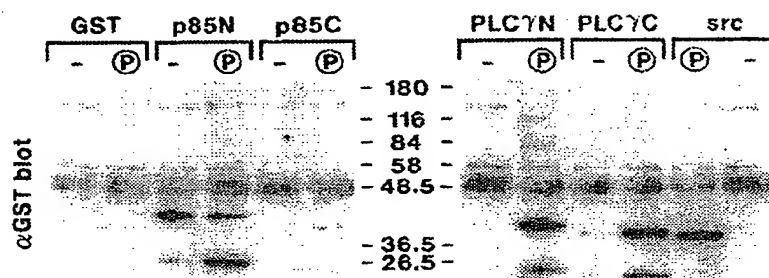
FIGURE 14



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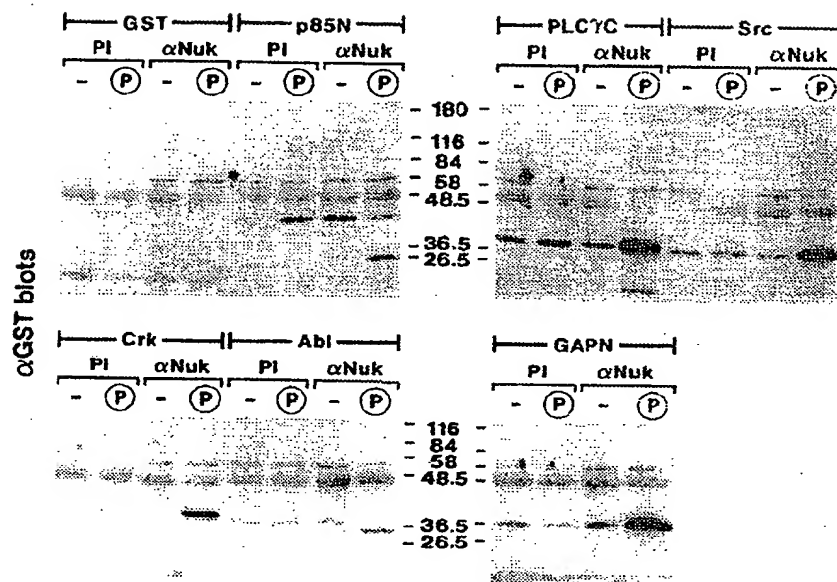
FIGURE 15



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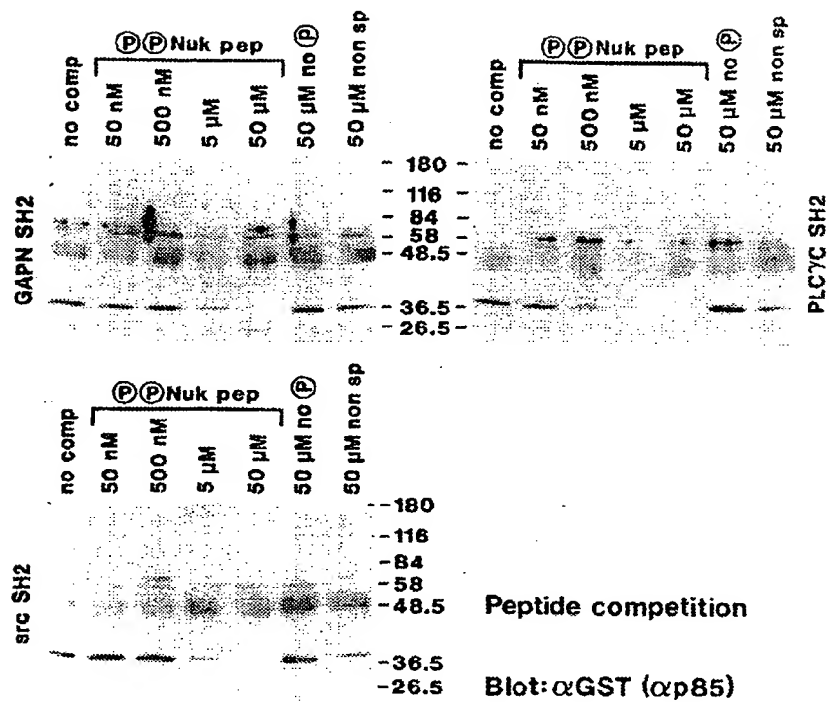
FIGURE 16



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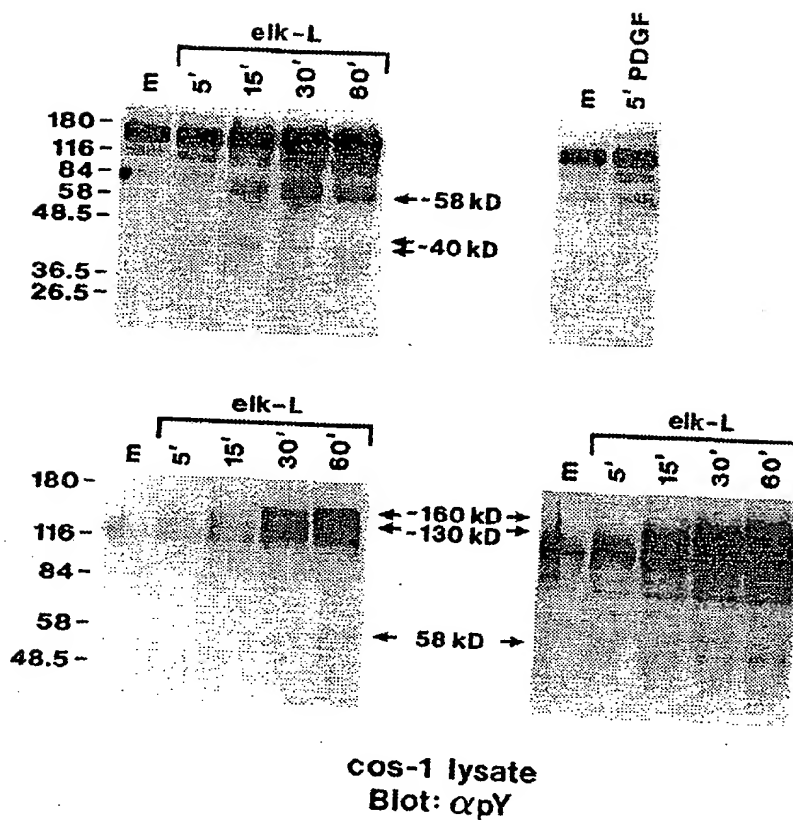
FIGURE 17



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FIGURE 18



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INTERNATIONAL SEARCH REPORT

Internatic Application No
PCT/CA 95/00254

A. CLASSIFICATION OF SUBJECT MATTER

IPC 6 C12N15/54 C12N9/12 C12N5/10 C12Q1/48 C07K16/40
C12N15/00 A01K67/027

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 6 C12N C12Q

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	EMBL RODENTS DATABASE, Accession no. L25890, sequence reference MMNRTK, November 6, 1993; M. HENKEMEYER: "Immunolocalization of the Nuk receptor tyrosine kinase suggests a role in segmental patterning of the brain and axonogenesis" see the whole document ---	1-8, 14, 15
X	ONCOGENE (1994), 9(4), 1001-14 CODEN: ONCNES; ISSN: 0950-9232, April 1994 HENKEMEYER, MARK ET AL 'Immunolocalization of the Nuk receptor tyrosine kinase suggests roles in segmental patterning of the brain and axonogenesis' see the whole document --- -/-	1-16

☒ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

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Date of the actual completion of the international search

19 September 1995

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Van der Schaal, C

INTERNATIONAL SEARCH REPORT

International Application No
PCT/CA 95/00254

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	<p>KEYSTONE SYMPOSIUM ON GROWTH AND DIFFERENTIATION FACTORS IN VERTEBRATE DEVELOPMENT, KEYSTONE, COLORADO, USA, APRIL 3-10, 1992. J CELL BIOCHEM SUPPL 0 (16 PART F). 1992. 106. CODEN: JCBSD7, HENKEMEYER M ET AL 'THE MURINE RECEPTOR TYROSINE KINASE NUK IS CONCENTRATED IN A SUBSET OF CELL-CELL JUNCTIONS DURING EMBRYOGENESIS.'</p> <p>see the whole document</p> <p style="text-align: center;">---</p>	1-16
A	<p>WO-A-93 00425 (INST MEDICAL W & E HALL) 7 January 1993</p> <p>see page 9, paragraph 3 - page 10, paragraph 1</p> <p style="text-align: center;">---</p>	9-12
A	<p>CELL,</p> <p>vol. 75, 17 December 1993</p> <p>pages 1157-1167,</p> <p>S.LYMAN ET AL 'Molecular cloning of a ligand for the flt3/flk-2 tyrosine kinase receptor'</p> <p>cited in the application</p> <p>see page 1157, right column, paragraph 3 - paragraph 3</p> <p style="text-align: center;">---</p>	9-12
A	<p>EMBO JOURNAL,</p> <p>vol. 9, no. 10, 1990</p> <p>pages 3279-3286,</p> <p>A. KAZLAUSKAS AND J. COOPER</p> <p>'Phosphorylation of the PDGF receptor beta subunit creates a tight binding site for phosphatidylinositol 3 kinase'</p> <p>see the whole document</p> <p style="text-align: center;">-----</p>	13

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

PCT/CA 95/00254

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO-A-9300425	07-01-93	AU-B- 655299	15-12-94
		EP-A- 0590030	06-04-94
		JP-T- 6508747	06-10-94
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